2020

Pathogenesis and control of Senecavirus A in swine

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Pathogenesis and control of Senecavirus A in swine

by

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A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Veterinary Microbiology

Program of Study Committee:
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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2020

DEDICATION

To J.D. and H.R. for moving with me to Iowa and their unwavering support of my pig filled dreams.

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NOMENCLATURE

ABSL-2	Animal Biosafety Level-2
ANTXR1	Anthrax Toxin Receptor 1
ATCC	American Type Culture Collection
BID	Twice A Day
CPE	Cytopathic Effect
Ct	Cycle Threshold
CSFV	Classical Swine Fever Virus
DPI	Days Post Infection
DPC	Days Post Challenge
DMEM	Dulbeecco's Modified Eagle's Medium
ELISA	Enzyme-Linked Immunosorbent Assay
ETNL	Epidemic Transient Neonatal Losses
FAD	Foreign Animal Disease
FBS	Fetal Bovine Serum
FMD	Foot-and-Mouth Disease
FMDV	Foot-and-Mouth Disease Virus
GC	Genomic Copies
IFA	Immunofluorescence Assay
IHC	Immunohistochemistry
INF	Interferon
IRES	Internal Ribosome Entry Site
IRF	Interferon Regulatory Factor

ISG	Interferon-Stimulated Gene
ISH	In-Situ Hybridization
IVD	Idiopathic Vesicular Disease
MEM	Minimum Essential Medium
MID	Minimum Infectious Dose
NADC	National Animal Disease Center
NGS	Next-Generation Sequencing
NVSL	National Veterinary Services Laboratory
OIE	World Organization for Animal Health
ORF	Open Reading Frame
PFU	Plaque-Forming Units
PIVD	Porcine Idiopathic Vesicular Disease
PCR	Polymerase Chain Reaction
PRR	Pathogen Recognition Receptor
RdRP	RNA Dependent RNA Polymerase
RIG-1	Retinoic acid-Inducible Protein-1
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RT-qPCR	Real Time Reverse Transcriptase-Polymerase Chain Reaction
SCLC	Small Cell Lung Cancer
SD	Standard Deviation
SEM	Standard Error of the Mean

ST	Swine Testicular
SVA	Senecavirus A
SVDV	Swine Vesicular Disease Virus
SVV	Seneca Valley Virus
SVV-001	Seneca Valley Virus-001
TCID	Tissue Culture Infectious Dose
TEM-8	Tumor Endothelial Marker-8
US	United States
UTR	Untranslated Region
VN	Virus Neutralization

ACKNOWLEDGMENTS

First, I sincerely appreciate all my friends and family who have supported me through all my further education. Second, I am grateful that Dr. Kelly Lager, my Co-major Professor, took a chance on a desperate fourth year veterinary student looking for a job and provided me with an amazing research opportunity. He has been an invaluable mentor and helped me realize my passion for swine virology. Third, I would like to thank Dr. Cathy Miller, my other Co-major Professor, for being there for every email and keeping this train on track. Finally, I would like to thank my committee members Dr. Phillip Gauger, Dr. Daniel Linhares, and Dr. Locke Karriker for their support and swine wisdom throughout my graduate work.

I would like to thank all those involved in the ARS Emerging and Endemic Viral Disease of Swine group and my collaborators Dr. Kyoung-Jin Yoon and Dr. Baoqing Guo from Iowa State University for their unending support of the research presented here. In the end, none of this would have been possible without the love and support from my family.

ABSTRACT

Senecavirus A (SVA) is a nonenveloped, single-stranded, positive-sense RNA virus in the family *Picornaviridae.* It was first discovered as a cell culture contaminant in 2002 but had been identified in US swine samples dating back to the late 1980s. Since swine were presumed to be the natural host, pigs were experimentally inoculated, but did not develop any specific clinical disease. Prior to 2015, SVA was sporadically detected in US swine associated with various clinical histories. In 2015, cases of vesicular disease and increased neonatal mortality were observed in Brazil and subsequently in the US. SVA was consistently detected in affected animals. Although previous attempts to reproduce disease with SVA in the past were unsuccessful, experimental inoculation studies performed with contemporary isolates demonstrated SVA was a causative agent for vesicular disease in swine.

SVA is now included in the differential list of etiologic agents that can cause vesicular disease in swine. This list includes foot-and-mouth disease virus (FMDV), which is a notifiable disease, so when vesicular lesions are observed in FMDV-free countries an investigation must be initiated to rule out FMDV. SVA has now been found across the Americas and Asia, and it appears the ecology of this virus has changed from sporadic infections to an endemic disease that does not induce severe clinical disease; but, its presence does have a significant impact since each case needs to be investigated as if it was a FMDV case. Thus, control and prevention measures are critical to reducing the spread of SVA in the global swine industry.

Due to the difficulty of reproducing clinical disease in past experimental challenges and the timing of SVA outbreaks in the field with stressful situations, there was speculation that stress may be a factor in the development of vesicular lesions. To study the impact of stress, an immunosuppressive dexamethasone regime prior to SVA inoculation was administered. Clinical

presentation and infection dynamics were similar in pigs treated with dexamethasone and those not treated prior to challenge. Another hypothesis for the lack of lesions in early experimental challenges was older isolates were less pathogenic than contemporary isolates. Vesicular lesion development and viral shedding in swine were compared between three SVA strains isolated prior to the 2015 outbreak and three strains isolated during 2015. The majority of animals in all groups developed vesicular lesions, and serum had cross neutralizing titers against all viruses. Sequencing and analysis of isolates used in the study found amino acid differences between the isolates in prominent loop structures of the capsid that may be involved with virus receptor binding and host immune response.

Due to the clinical similarities between SVA and FMDV, control and prevention of SVA infections are a priority. Understanding the minimum infectious dose (MID) of SVA could provide insights into the infectivity of virus found in the environment and how the virus is spread. Finishing pigs and neonates were used to determine the MID of a 2011 SVA isolate using intranasal and oral challenge routes respectively. In this study, finishing pigs had a MID of $10^{3.1}$ $TCID_{50}$/mL and neonates $10^{2.5}$ $TCID_{50}$/mL. Although there were differences in infectious dose, fewer dilutions were tested in finishing pigs, which may provide a less precise estimate compared to the neonates. Another control measure for SVA could be vaccination. An inactivated vaccine was tested in weaned pigs and mature sows. The vaccine prevented the development of clinical signs and viremia as well as reduced rectal shedding in pigs. In addition, piglets suckling immunized dams had sterilizing immunity against SVA challenge. Overall, a better understanding of the pathogenesis of SVA in swine can help improve control and prevention measures to reduce the burden of SVA infection on the swine industry.

CHAPTER 1. GENERAL INTRODUCTION

Background

The family *Picornaviridae* is a large family of RNA viruses consisting of over fifty genera that are capable of infecting many species of veterinary importance (ICTV 2019) (1). There are limited reports of picornavirus infection in companion animals, but there is a long history of picornaviruses in livestock species (2, 3). In 1897, Loeffler and Frosch discovered a filterable agent caused foot-and-mouth disease (FMD) in cattle, which was the first description of an animal virus, foot-and-mouth disease virus (FMDV) (4-6). Disease manifestation from picornavirus infection in livestock can include a wide range of clinical signs including those involving the central nervous system (porcine teschovirus), digestive system (bovine enterovirus), and respiratory tract (equine rhinitis virus) (7-11). In addition, there is large diversity in disease severity caused by infections (12). Though some picornaviruses can cause significant clinical disease, due to their ubiquitous prevalence, a majority of infections are asymptomatic (7).

Swine can be infected by several viruses found in the family *Picornaviridae* (13). Two of these viruses, FMDV and swine vesicular disease virus (SVDV), cause clinically identical vesicular disease, which involves blister-like lesions that form on the coronary bands and snout. Porcine enteroviruses circulate worldwide in domestic pigs, and infections are often asymptomatic; but, depending on the virus serotype and conditions of infection, they can cause clinical signs including neurologic disorders, reproductive failure, and enteric disease (14). Due to the diversity of porcine enteroviruses, they have now been reorganized into three genera within the family *Picornaviridae*: *Enterovirus*, *Teschovirus*, and *Saplovirus* (15). A recent addition to the picornavirus family that infects swine was named Senecavirus A (SVA), which is

the only member of the genus *Senecavirus* and shares closest sequence identity to the genomes of those viruses in the *Cardiovirus* genus (16).

SVA was originally identified as a cell culture contaminant, but subsequent research has demonstrated SVA to be a causative agent for vesicular disease in swine, which is clinically identical to FDMV and SVDV (16, 17). In countries participating in the World Health Organization (OIE), FMDV is a reportable agent. The US is FMDV-free and when a vesicle is observed in livestock, a foreign animal disease (FAD) investigation must be initiated to rule out FMDV. Since the discovery of SVA in the US, it has been identified in other countries including Brazil, Canada, and China (18-20). Understanding the pathogenesis of SVA and molecular evolution can aide in the development of control measures to prevent further spread of the virus.

Research Objectives

Unsuccessful attempts to reproduce clinical disease experimentally and isolation of SVA from asymptomatic animals led to questions about why some animals develop vesicular lesions and others do not (21, 22). Hypotheses have included pathogenicity differences between SVA strains, host factors, and the requirement of a co-factor (23). Vesicular disease in the field due to SVA often coincided with stressful scenarios, such as marketing of finishing hogs and parturition at sow farms. Thus, research was performed to determine the role of stress in SVA clinical disease progression and infection dynamics in nursery pigs. To test the hypothesis of pathogenicity differences in SVA isolates, three historical and three contemporary isolates were chosen for an animal study. Vesicular lesion development, viremia, and viral shedding in oral and rectal swabs was compared between groups. Serum collected from pigs at necropsy was also used to analyze cross-neutralizing titers against all isolates. Finally, sequences were analyzed for differences in prominent loops of the structural proteins involved with receptor binding.

Sow slaughter facilities have continued to be a source of FAD investigations due to vesicular lesion detection. Co-mingling of animals from different sources and passing them through continuous flow buying stations may be contributing to virus transmission and chronic reporting of vesicular cases (24). Understanding the infectious dose of SVA and viral load in the environment could help direct efforts to reduce virus transmission. This work used a 2011 SVA isolate to determine the minimum infectious dose in both finishing pigs and neonates. Another goal was to correlate polymerase chain reaction (PCR) cycle threshold (Ct) values to virus titration in cell culture, which can aide in interpretation of environmental samples and the effectiveness of cleaning and disinfection procedures. Vaccines also play a crucial role in disease management in swine. The final objective of this work was to evaluate the efficacy of an inactivated SVA vaccine in both weaned pigs and sows. In addition, passive maternal immunity was tested by challenging piglets suckling immunized dams.

Implications

Knowledge about the pathogenesis of SVA in swine can provide many benefits to the swine industry. Understanding how long the virus can be detected in various sample types after infection can aide in choosing the correct samples to collect for diagnosis. In addition, determining the infectious dose of SVA and the duration of virus shedding can help determine measures to control virus spread between animals. Prevention of SVA infection and disease with an efficacious vaccine would improve swine welfare, minimize SVA transmission, and reduce the burden of FAD investigations.

Dissertation Organization

This dissertation contains seven chapters. Chapter 1 is a general introduction and rationale for the research performed. Chapter 2 is a literature review of *Senecavirus A* (SVA). Chapter 3 is a modified manuscript published in BMC Veterinary Research studying the effect of

stress on SVA infection dynamics and vesicular lesion development by comparing pigs treated with an immunosuppressive dexamethasone regime prior to SVA inoculation to pigs that were not treated prior to challenge. Chapter 4 is a modified manuscript that is under review in Veterinary Microbiology comparing SVA infection dynamics of three historical SVA isolates collected prior to 2015 and three contemporary isolates from 2015 and the cross-neutralizing antibody titers. In addition, nucleotide sequences from the isolates were compared and predicted amino acid sequence differences from significant loop structures on the capsid were analyzed. Chapter 5 is a manuscript prepared for submission to PLoS One reporting the minimum infectious dose of SVA required to infect finishing pigs and neonates. Chapter 6 is a manuscript prepared for submission to Vaccines evaluating the efficacy of a whole-virus SVA inactivated vaccine against SVA challenge in weaned pig and sows. Passive maternal immunity provided by vaccinated sows was also tested by piglet challenge. Chapter 7 summarizes the research in this dissertation and its implications, as well as future directions for research.

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CHAPTER 2. LITERATURE REVIEW

Picornavirus Biology

Picornaviruses are small, non-enveloped, single-stranded, positive sense RNA viruses with genomes ranging from approximately 7 to 10 kilobases (kb) (1). The 5' untranslated region (UTR) links to a small peptide VPg, which acts as a primer during RNA synthesis, and the 3' UTR is followed by a polyA tail (2, 3). Both UTRs have significant secondary structures with the 5'UTR containing an internal ribosome entry site (IRES), which is critical to initiate cap-independent translation required by picornaviruses (4, 5). In between the 5' and 3' UTRs lies a single open reading frame (ORF) encoding a polyprotein processed post-translationally by viral proteases into four structural proteins (VP1, VP2, VP3, and VP4) and seven nonstructural proteins (2A, 2B, 2C, 3A, 3B, 3Cpro, 3Dpol) (6). Some genera also contain the Leader protein (L-pro), thus resulting in the conserved picornavirus L-4-3-4 layout, with the structural proteins followed by the nonstructural proteins (7). They have an icosahedral capsid approximately 30 nm in diameter that is composed of 60 protomers made up of each of the four structure proteins (VP1, VP2, VP3, and VP4) (8). VP1-3 reside on the outer surface of the capsid with VP4 located internally (9, 10). L-pro, 2A, and 3Cpro are viral proteases involved in cleaving both the viral polypeptide as well as host proteins (9).

To gain entry into the cell, picornaviruses bind to a cell surface receptor, which leads to receptor mediated endocytosis (10, 11). Subsequent destabilization of the capsid, through the loss of VP4 and creation of a channel thought to involve VP1, leads to the release of the genome from the acidic environment of the endosome into the cytoplasm (8, 12). Since the genome is positive sense, translation can begin immediately to produce mature viral proteins (5). Eventually, there is a transition from translation to transcription, and genome replication by the

RNA-dependent RNA polymerase (RdRP, 3Dpol) occurs in viral replication complexes formed by viral modification of host cell membranes (13). Replication starts with negative strand synthesis that serves as a template for positive strand synthesis, which can be used for further protein synthesis or be packaged into new virions. VP0, the precursor of VP4 and VP2, along with VP1 and VP3, form protomers and pentamers that come together with the viral genome to form the provirion (14). The final step of virion formation involves the cleavage of VP0 into VP4 and VP2, which is suspected to be autocatalytic (15, 16). This process has been reported to change the capsid conformation and is critical for an infectious mature virion (17). Mature virions are typically thought to be released through cell lysis, but recent work has also shown viruses escaping via lipid membrane enclosed particles or vesicles (18, 19).

Type 1 interferons are an important part of the innate immune response to control picornavirus infection as well as neutralizing antibodies from the adaptive immune response (20). Picornaviruses have evolved many mechanisms to counteract and evade these host immune responses. Viral proteases have been shown to suppress interferon (IFN) production and block IFN induction of interferon-stimulated genes (ISGs), thus inhibiting the early steps of the innate immune response (21). Interference with host pathogen recognition receptors (PRRs), like retinoic acid-inducible protein 1 (RIG-1), has also been reported; and without innate immune recognition, the more specific adaptive immune response is delayed (22). Picornaviruses are known for the rapid shutdown of host translation, resulting in the preferential translation of viral RNA and reduced cellular transcription (20, 23). They are also known to modulate cellular pathways for apoptosis and autophagy to promote viral replication and dissemination (11). Finally, the low fidelity and absence of excision activity for proofreading of picornavirus RdRPs

can result in higher mutation rates and the ability to more rapidly adapt to defenses mounted by the host (24).

Senecavirus A Biology

As a member of the family *Picornaviridae*, SVA has one ORF encoding a large polyprotein that is then cleaved into 12 mature proteins in the standard picornavirus L-4-3-4 layout (25). The three major protein regions are P1 (VP1, VP2, VP3, and VP4), P2 (2A, 2B, 2C), and P3 (3A, 3B, 3C, 3D) (26). The single ORF is flanked by a long 5'UTR (666 residues) and short 3'UTR (71 residues) followed by a poly(A) tail resulting in a genome ~7.3 kb (27). SVA has a type IV IRES in the 5'UTR with a predicted secondary structure most closely related to classical swine fever virus (CSFV) (28). There is evidence that IRES elements can move between genomes, thus leading to speculation of recombination during a co-infection event of CSFV and SVA in swine (28, 29).

The capsid consists VP1, VP2, VP3, and VP4 with lengths of 265, 286, 241, and 74 residues, respectively (30). Prominent loop structures in the structural proteins of the capsid for SVA have been identified: BC loop of VP1 (residues 50-74), Loop II or CD loop of VP1 (residues 94-109), GH loop of VP1 (residues 185-215), "the puff" of VP2 (residues 172-200), and "the knob" of VP3 (residues 57-73) (30). These structures on the capsid can help determine cell tropism as well as antigenic sites that elicit immune responses to infection (31). Anthrax toxin receptor 1 (ANTXR1), also known as tumor endothelial marker 8 (TEM8), has been demonstrated to be a receptor for SVA interacting mainly with the BC loop and Loop II of VP1, "the puff" of VP2, and "the knob" of VP3 (32-34). Binding of the virus to the receptor causes structural changes in both VP1 and VP3 (35). In addition, multiple linear B-cell epitopes have been identified for both VP1 and VP2 (36).

The functions of the nonstructural proteins have mostly been assumed, based on knowledge gained from past picornavirus research. For example, 2A has a predicted ribosome-skipping function, and 2C is similar to a helicase that assists with RNA synthesis (37). Other proteins, including 3C and 3D, have more conserved motifs with other known picornaviruses serving as a proteinase and RdRP, respectively (38). Fortunately, research groups have successfully generated infectious cDNA clones of full-length wild-type SVA from both the United States (US) and China (39-41). A reverse genetics system has many benefits including further characterization of SVA biology and pathogenesis, as well as vaccine development (39, 40). In addition, SVA has been found to replicate in multiple cell lines, such as swine testicular (ST) cells, porcine kidney cells, baby hamster kidney cells, and a multitude of human cancer cells lines (e.g. H1299) thus making the virus easy to work with in cell culture (42, 43).

Cells generate a type 1 IFN response as a first line of defense against SVA infection, and SVA has shown sensitivity to IFN-β (44-46). RIG-1, a PRR, was shown to be essential to activate the type 1 IFN cascade in SVV infected cells (46). To combat this, 2C and 3C induce the degradation of RIG-1, subsequently reducing IFN-β production (47). 3CPro protease activity, including reducing interferon regulatory factor (IRF) 3 and IRF7 protein levels and deubiquitinating activity, helps SVA evade the host type 1 interferon response by reducing the transcription of IFN-β, IFN-α, and ISG54 (48, 49). In addition, 3Cpro cleaves host adapters, including mitochondrial antiviral signaling (MAVS), which also leads to the disruption of PRR-mediated IFN production (45). Finally, 3CPro was also associated with the cleavage of nuclear factor-kappa B (NF-κB), along with 2C activating caspase-3, suggesting viral involvement in promoting cellular apoptosis for dissemination of virus (50, 51). Additional work in cell culture

has demonstrated SVA induces autophagy to promote virus production, and exosomes can mediate intercellular transmission for viral spread (52, 53).

One interesting aspect of SVA biology is its cytolytic potential in neuroendocrine and pediatric tumors (54, 55). Cell lines from small-cell lung cancer (SCLC) and solid pediatric cancer were 10,000-fold more sensitive to SVA cytolytic activity than cell lines from normal human tissue (54). TEM8 is upregulated in some tumor cell populations, which could explain SVA selectivity for infecting tumor cells (33). Initial studies using mice as human models showed promise in treating SCLC, medulloblastoma, retinoblastoma, and pediatric malignant glioma (56-59). The oncolytic virus was moved forward to Phase 1 trials to treat neuroendocrine tumors in both adults and children; although there was evidence of antitumor activity, neutralizing antibody production may limit utility (60, 61). Phase II trials in adults with late-stage SCLC did not benefit from treatment (62).

Clinical History

SVA was first discovered in 2002 at a laboratory in Maryland as a cell culture contaminant in PER.C6 cells and was named Seneca Valley virus-001 (SVV-001) (25). It was speculated the contamination could have been introduced by either porcine trypsin or fetal bovine serum, both commonly used in cell culture (54). The National Veterinary Services Laboratory isolated twelve picornavirus-like viruses between 1988 and 2005 from swine exhibiting a variety of clinical signs and from multiple states across the US (63). Sequencing highlighted the close relationship of these isolates with SVV-001, and neutralizing antibodies were found in swine serum samples supporting swine as a natural host. Interestingly, serum samples from cattle also had neutralizing antibodies (63). Two of these historical isolates were used to inoculate pigs, but they did not develop any specific clinical disease (63).

Market weight pigs being transported from Canada to the US in 2007 arrived with a majority of animals presenting with lameness and some with vesicular lesions on the snouts and coronary bands (64). Samples from these animals were tested for the top differentials for swine vesicular disease: FMDV, SVDV, vesicular stomatitis (VS), and vesicular exanthema of swine (VES). Results were negative, so the animals were diagnosed with idiopathic vesicular disease (IVD), though samples from these animals did test positive for SVA by reverse transcriptase polymerase chain reaction (RT-PCR) (64). Subsequently, a boar in Indiana that had vesicular lesions in the oral cavity, on the snout, and on the coronary bands tested positive for SVA (65). Since SVA had also been found in healthy swine, the case was also considered IVD. Due to evidence that SVA infected swine, Yang et al. developed a competitive enzyme-linked immunosorbent assay (ELISA) tested using serum generated from experimentally inoculated pigs (42). Inoculated pigs did not develop clinical disease, though they did generate an antibody response. Thus, experimental infection with SVA failed to reproduce any consistent clinical disease, but evidence from field cases supported an association of SVA infection with vesicular disease in swine.

Beginning in late 2014, reports of vesicular lesions in swine, along with an increase in neonatal mortality observed in piglets less than a week of age, were spreading across the swine producing regions of Brazil (66). The mortality observed in the piglets was given the name epidemic transient neonatal losses (ETNL), and piglets displayed inconsistent clinical signs prior to death including lethargy, wasting, neurologic signs, and diarrhea (66). Samples collected from these cases tested negative for the classical causes of vesicular disease in swine but did test positive for SVA (67). In the summer of 2015, similar cases of vesicular disease were observed in swine in the US that also tested positive for SVA with a 97.7-98.0% nucleotide identity to the

isolates from Brazil (68, 69). In addition, there were similar reports of ETNL at sow farms experiencing outbreaks of SVA and vesicular disease (70).

Overwhelming evidence from the field supported SVA as a causative agent for vesicular disease in swine; however, prior to these outbreaks, no group had been able to produce specific clinical disease after experimental inoculation. Two groups used a 2015 SVA strain isolated from a vesicular disease case in finishing pigs from South Dakota and were able to experimentally induce vesicular lesions on coronary bands and the snouts of both 9-week-old pigs (71) and 15-week-old pigs (72). In addition, another group using a different 2015 SVA isolate was able to reproduce vesicular lesions in 3-week-old pigs (41). Thus, there now was evidence, both experimentally and from the field, that SVA is a causative agent for vesicular disease in swine. Since multiple groups were able to experimentally reproduce disease, questions remain as to why previous attempts with historical isolates were unsuccessful. Some areas of speculation include differences in pathogenicity of viruses, susceptibility of the host, or the requirement of a novel co-factor.

Epidemiology

Shortly after the 2015 outbreak began, a diagnostic laboratory from the US tested oral fluids from 441 sample submissions representing 25 states and 5 samples tested PCR positive for SVA equaling an incidence of ~1% (73). During 2017, 444 diagnostic submission samples (serum, swabs, oral fluids, and tissues) reported a 5.4% positive rate; but if the 225 serum samples were removed, the positive rate increased to 10.5% (74). In addition, prevalence of SVA antibodies in swine was measured from samples collected in 2016 from 3,654 growing pigs and 2,433 sows using both an ELISA and immunofluorescence assay (IFA). They estimated the seroprevalence of growing pigs was 12.2% and of sows was 34% (75). Scientists in Brazil compared seroprevalence of antibodies against SVA in serum samples collected prior to the

outbreaks in 2014/2015 and after those outbreaks. Samples prior to the outbreak were negative for antibodies while 34.6% of post-outbreak samples were positive, supporting that SVA had not been circulating in Brazil prior to 2014 and that seroprevalence was similar to sow farms in the US (76).

Although the first SVA cases reported in the US in the summer of 2015 involved pigs from county fairs and late finishing pigs in the Midwest, the virus was also quickly identified at sow farms reporting increases of neonatal mortality ranging from 30-70% along with a diverse range of adult animals exhibiting vesicular disease (77). One study reported that 2/6 SVA-affected breeding herds did not report vesicular lesions in sows (78). In Brazil similar findings were reported consisting of neonatal mortality ranging from 5-60% and mixed reports on the number of affected farms that also observed vesicular lesions on the sows (38). A study comparing clinically affected sows to non-clinically affected sows on a sow farm experiencing an SVA outbreak demonstrated similar PCR positive samples and antibody responses between both groups (79). Therefore, not all animals infected with the virus develop vesicular disease and affected farms may be under reported, thus contributing to the spread of SVA.

Epidemiologic investigations assigned employee entry, carcass disposal, and cull sow removal as high-risk events for SVA introduction to a farm, but also mentioned rodents, feed delivery, and semen entry as high risk (78). Live virus has been isolated from environmental samples (e.g. dust on fans, farm hallways, the ground outside, and a mortality tractor bucket), mouse feces, and mouse small intestine from an affected farm; but it was also detected via PCR in flies collected from both affected and unaffected farms, thus providing evidence that these pests could play a role in the spread of SVA between farms (80). Recently, feed has been suspected as a vector to transport virus between countries (81, 82). Dee et al. demonstrated that

SVA remained infectious in most feed ingredients tested in a simulated Trans-Pacific journey and was shown to be the most stable of all the viruses studied (83). SVA has been detected in feed ingredients and complete feed collected from two feed mills in Brazil, but further research will need to be performed to determine the risk of transmission through feed (84). In addition, environmental samples from an assembly yard in Canada have tested positive for SVA (85). The trafficking of animals to slaughter plants daily provides abundant opportunity for virus spread between slaughter plants and trucks and back to farms or collection points. Trucks have been shown to play a role in the spread of viruses as demonstrated by studies with porcine epidemic diarrhea virus (86, 87). Recently, risk factors for SVA positive pigs arriving at a slaughter plant included suppliers that raised pigs indoors and suppliers with multiple type sites (88).

Semen positive for swine viruses, including CSFV, porcine respiratory and reproductive syndrome virus (PRRSV) and pseudorabies virus (PRV), has been experimentally shown to infect sows (89). Semen has tested PCR positive for SVA, but additional research will need to be performed to determine the potential for viral transmission during breeding (70). Muscle meat cuts were tested to understand if retail meat could be a risk of SVA transmission. Only 1.1% (2/190) were PCR positive for SVA, but both samples were virus isolation negative suggesting that retail meat is a low risk for SVA transmission (90). Little objective data has been collected regarding cull sow movements; but one study found a significant number of sows entered multiple collection points prior to reaching a slaughter facility and traveled on average over 240 km from their site of origin, making the cull sow network a likely area of pathogen transmission (91). Samples from collection points in North Carolina from animals headed to slaughter tested positive for the presence of SVA by PCR suggesting that SVA does circulate in secondary and cull sow markets and mostly likely contributed to the spread of SVA nationally in the US (73).

Since the 2015 outbreaks reported in Brazil and the US, vesicular disease cases due to SVA have been found across the globe including China (92), Canada (85), Colombia (93), Thailand (94), and Vietnam (95). In March of 2015, SVA was discovered and isolated in China with farms reporting vesicular lesions in sows and acute death in neonates similar to Brazil (96). In October of 2015, sows transported to the US from Canada had vesicular lesions on arrival, which initiated a foreign animal disease (FAD) investigation. Tracebacks to the herd of origin detected SVA that was genetically similar to isolates found in China (85). In February of 2016, a breeding farm in Colombia broke with vesicular disease, and phylogenetic analysis of the SVA isolate clustered the Columbian strain with contemporary isolates from the US (98.5-98.9% nucleotide identity) rather than strains from Brazil (93). SVA was first detected in Thailand in October of 2016 with lesions in market-weight pigs. At a genomic level, this virus was most closely related (98.2%) to the first Canadian strain from 2011 (94). Research has suggested the SVA introduction to Thailand may not have been recent, but that the virus had evolved in the Thai swine population and remained undetected due to the presence of other pathogens that cause similar clinical disease, like FMDV (97). Finally, in January of 2018, a group of pigs from Vietnam diagnosed with FMDV also tested positive for SVA, and the sequence shared high homology with isolates collected from China in 2015 and 2016 (95). This case and others highlight the difficulties faced by countries with FMDV and SVA co-circulating in swine herds to understand virus spread and mount control responses to each virus.

Brazilian isolates appear to have originated from a common source, since they are genetically similar and group together in a clade separate from most US isolates from the same time period (98). Similarly, isolates from the US also clustered together; and, in general, SVA isolates cluster according to geographic region (99). Surprisingly, early SVA isolates from China

(2015-2016) tended to cluster together near Canadian and Brazilian isolates, while isolates from 2016-2017 began to cluster within the 2015 US isolates (100-105). Of note, one report commented that earlier isolates more closely related to Brazilian isolates caused acute death in neonates, while the more recent strains clustering with the US isolates did not cause clinical disease in piglets (106). Clusters of Chinese isolates spread throughout SVA phylogenetic trees with little relationship to area or year of isolation could imply multiple introductions into China and/or undetected circulation and adaptation in Chinese swine herds (107, 108). The genetic distance of isolates between the US and Brazil was reported to be 2.71%, while the distance between the US and China isolates was 2.48% and 2.8% between Brazil and China isolates (99). The overall genetic divergence of contemporary isolates was 2.8%, but the genetic divergence between contemporary isolates (2011-2017) and historical isolates (1988-2010) was 6.32% (99). Another wave of outbreaks was reported in Brazil during the second half of 2018 in many of the same states that experienced outbreaks in 2015. Although the clinical disease presentation seemed more severe, phylogenetic analysis suggested the 2018 strains were not significantly different from those strains sequenced in 2015-2016 in Brazil (109).

There have been multiple reports of recombination events with Chinese SVA strains. A few events involving Chinese isolates from 2017-2019 showed parental strains from 2015 US isolates (110, 111). In addition, a Chinese isolate from 2018 was reported to have SVV-001 as a parental strain, and an isolate from 2019 was reported to have the 2016 isolate from Colombia as a parental strain (112, 113). Finally, there have also been reports of recombination events with Chinese isolates from 2016-2019 involving two Chinese parental strains (111, 114). Thus, there is evidence of recombination of SVA in China dating back to at least 2016. These events have been found to occur across the SVA genome including events in P1, P2, and P3 (112). SVA's

RdRP has been shown to play a central role in SVA replicative recombination, and mutation rate was linked to recombination rate (115). Though more research needs to done in this area with SVA, evidence of recombination events has been reported in many other picornaviruses, including FMDV, and can play a key role in virus evolution (116).

Pathogenesis and Immune Response

Vesicular lesions in swine can be found on the coronary bands, intradigital space, snout, lips, and tongue (117). Lesions can begin with erythematous areas or blanched areas of the skin progressing to vesicles with varying levels of fluid that rupture leaving an erosion on the skin that crusts over and resolves (72). Histologically, areas of separation between the dermis and epidermis with clefts are noted containing edema, fibrin, necrotic debris, and inflammatory infiltrates (neutrophils, lymphocytes, and plasma cells) (72, 118). Development of vesicular lesions on the coronary bands have been observed in as little as 48 hours in market-weight animals, but most animals develop vesicular lesions 3-6 days after experimental challenge and heal within 7-14 days (41, 71, 72, 119, 120). Snout lesion development has been described as delayed compared to the appearance of coronary band lesions and heals more quickly (119, 121). In addition, some studies have reported seeing less snout lesions compared to coronary band lesions (118, 122). Other clinical signs that have been reported intermittently in pigs inoculated with SVA include fever, lameness, lethargy, and decreased feed intake (26, 106).

Although most experimental infection studies with contemporary SVA strains have resulted in most animals developing vesicular lesions, field reports have described varying levels of incidence (67, 70, 78, 123). It is still not understood why some animals develop vesicular lesions and others do not (124). Exposure dose may play a role considering most experimental studies with swine have used inoculum doses between 10^7 and 10^8 $TCID_{50}$/mL, which may be higher than exposure levels in the field. Experiments with FMDV in swine have shown altered

infection dynamics and a shorter time to clinical signs with higher doses of inoculum (125, 126). There has also been speculation surrounding age-dependency of lesion development. One study, using a SVA isolate from China, inoculated pigs around 1, 2, and 3 months-of age. The oldest pigs were the only ones to develop vesicles on their coronary bands and snout, while the two groups of younger pigs did not develop any visible lesions (122). One hypothesis for differences in lesion development is density of the SVA receptor on susceptible cells in the epithelium of the coronary band and snout, which could be dependent on age or genetics of the pig (118).

Viremia after experimental challenge lasts between 1-10 dpi with peak levels around 2-4 dpi (41, 127, 128). Live virus has been isolated from serum on 2-3 dpi, but not later in infection (117, 128). Interestingly, it has been noted in recent experimental studies that not all challenged animals develop a viremia (120). Oral, nasal, and rectal swabs typically test PCR positive from 1-21 dpi, but there are sporadic positive samples out an additional week or more with oral and nasal swabs often testing PCR positive longer than rectal swabs (118, 121). Virus isolation (VI) performed on swab samples was successful on samples most reliably during the first week after inoculation, which coincides with peak RNA levels measured by PCR; although much less frequent, VI positive oral and fecal swabs have occurred out to 21 dpi (72, 117, 120).

Studies of SVA outbreaks in the field have supported observations from experimental studies. SVA shedding dynamics, during a natural infection at a sow farm over a 9 week period post onset of clinical signs, observed vesicular lesions for ~2 weeks and viremia for ~1 week, but rectal and tonsil swabs from both piglets and sows were PCR positive for SVA out 6 weeks post outbreak (123). Following an outbreak, a sow farm found rectal and tonsil swabs PCR positive on a breeding farm at least six weeks after the onset of clinical signs (79). For diagnostics in the field with unknown infection status, swabs may be a more reliable sample than serum due to the

greater longevity of SVA detection in swabs compared to serum. But, swabs of vesicular lesions are the best sample for the diagnosis of SVA, due to the high levels of virus compared to all other samples (119).

The role of stress and its effect on SVA pathogenesis and disease manifestation have been of interest following the first reports of SVA in the US involving show pigs and finishing pigs being transported to market. Since experimental inoculation in the past had been unsuccessful, it was hypothesized that stress may be a required co-factor for clinical manifestation of SVA infection. This hypothesis was also supported by the fact that the virus had been found in healthy pigs without vesicular disease (73, 124). One early experimental inoculation with SVA treated half the pigs with an immunosuppressive regime of dexamethasone prior to challenge. Both groups developed vesicular lesions and had similar infection dynamics, although a greater percentage of dexamethasone treated pigs showed clinical signs approximately 24 hours earlier than those not treated (118). Similarly, animals that had transportation stress prior to challenge developed lesions slightly earlier than animals with no stressor prior to challenge, but viremia, shedding dynamics, and neutralizing antibody response were similar between both groups (121). Thus, these studies support stress is not required for lesion development, but it may accelerate development.

There had also been speculation of stress causing SVA to recrudesce or to renew active replication. Recrudescence has been reported to occur in other viruses that infect swine including PRV, a herpesvirus that infects swine (129, 130). A field study observed an increase in the percentage of piglets positive for SVA in serum at weaning almost 3 weeks after the virus had been cleared from the serum of most piglets (123). An unconfirmed field report suggested that pigs without lesions present during marketing showed up to the slaughter plant ~12 hours later

with lesions. Lesions were not observed on other animals from the farm of origin leading to speculation of recrudescence. Experimental work to test this theory challenged three groups of pigs with SVA and 46 days after infection applied a stressor event: transport stress, dexamethasone treatment, or parturition. No lesions were observed after the stressor, but intermittent viremia and shedding was detected in all groups (121). Of note, shedding was still observed in some animals at the stressor event from the initial SVA challenge (121).

The extended shedding seen in some animals infected with SVA could be attributed to persistence of the virus in tonsils. Live virus has been isolated from a tonsil 60 days after initial challenge, and in situ hybridization localized the virus to both tonsillar epithelial cells as well as lymphoid tissues (121). Double-stranded RNA (dsRNA) was detected by IFA in tonsils indicating a potential mechanism for persistence that has been shown for other viruses including PRRSV that can persist in the germinal centers of lymphoid tissues as dsRNA (131, 132). In addition, dsRNA is also a product of positive-sense RNA viral replication, so the dsRNA could also represent continued replication of SVA in the tonsillar tissue. Sows that farrowed ~46 days after initial exposure to SVA were able to transmit virus to their piglets supporting continued replication in animals long after the resolution of clinical signs (121). Although piglets were found positive for SVA, as with other experimental studies, the piglets did not demonstrate any clinical signs.

Piglets in Brazil have been reported to have ulcerative lesions on the snout, tongue, and coronary bands in SVA affected farms; however, those lesions have not been reported frequently in piglets in the US (133, 134). Brazil also had more reports of neurologic disease in neonates, which was supported by immunolabeling of SVA found in the choroid plexus of the brain and the surrounding endothelium cells of blood vessels of piglets that died on SVA affected farms

(133). In addition, piglets submitted to a diagnostic lab in Brazil for ETNL had atrophic enteritis with positive immunolabeling in apical enterocytes as well as transmission electron microscopy evidence of viral particles similar in size and morphology to that of picornaviruses in the apical enterocytes (135). SVA was also detected in urinary epithelium by immunohistochemistry (IHC) with ballooning degeneration of the transitional epithelium (136). Histology and IHC demonstrated a multi-systemic infection of SVA in piglets; but quantification by PCR had demonstrated that the lymphoid organs have the highest levels of virus, which has also been observed in older swine after experimental challenge (118, 137). Lesions and virus in the urinary and enteric tracts suggest that urine and feces could be a mode of horizontal transmission of SVA. IHC detection of SVA in tissues from 1-2 day old piglets also suggests vertical transmission of the virus from sows (136).

Due to the inability of early studies to reproduce clinical disease with experimental SVA challenge, it was hypothesized that older isolates were less pathogenic than contemporary SVA isolates (118). One study reported that pigs challenged with SVV-001 did not develop vesicular lesions but did replicate the virus and develop an antibody response; while the group challenged with a 2015 isolate developed clinical disease and had greater levels of SVA RNA in acute serum and swab samples, except fecal swabs (120). Both groups developed cross-neutralizing antibodies and cross-neutralizing T-cell responses suggesting conserved antigenic determinants (120). Another group compared the pathogenicity of two contemporary Chinese isolates (2016 vs 2017) and found one isolate to be more pathogenic in pigs than the other, with the 2016 most closely related to Canadian isolates not causing vesicular disease in a group of finishing pigs (127). Recent cell culture work with 5 different Chinese isolates located in different SVA phylogenetic clusters also showed small differences in viral growth kinetics in ST cells (138).

Therefore, some SVA isolates have not caused vesicular disease in challenged pigs, and more research is necessary to correlate SVA genomic differences with pathogenic differences in SVA strains.

Neutralizing antibody titers have been measured in pigs as early as 3-5 days after experimental infection, which has been correlated with VP2- and VP3-specific IgM responses (41, 72, 119, 128). This rise in neutralizing antibodies corresponds with the decline in viremia. IgG antibody response to infection follows IgM with titers beginning around 10 dpi (72, 118). VP2-specific IgG antibodies were detected longer than VP1 and VP3 (128). Neutralizing antibodies have been found in animals up to 5 months after initial exposure, but further research must be performed to determine the protective titer. CD4+ helper T cells, critical for antibody production, were detected by 7 dpi, while CD8+ and CD4+ CD8+ T cells (effector/memory) increased after 10 dpi (128). VP2-specific responses were highly suggestive that VP2 contains important B-cell and T-cell epitopes (128).

Diagnostics

PCR is an invaluable tool for virus detection and SVA diagnosis. SYBR Green and TaqMan-based real time RT-PCR assays have been developed with probes targeting different regions of the virus, including VP1 and 3D (139-141). In addition, a nested-PCR assay has been developed to amplify a fragment of VP1, which was able to identify SVA RNA in samples considered negative by RT-PCR (142). A real time RT loop-mediated isothermal amplification (real time RT-LAMP) assay was developed to provide a cheaper option for SVA detection (143). The RT-LAMP procedure has also been combined with a lateral flow dipstick for rapid visualization of results (144). Finally, a RNA RT-droplet digital PCR (RT-ddPCR) was developed that allows quantification without the need for standard curves and is resistant to inhibitors present in different sample types (145).

The ability to differentiate between viruses that cause vesicular disease in swine is important since they are clinically indistinguishable. This is especially significant for FMDV, since it is on the World Organization for Animal Health (OIE) list of notifiable diseases (146). To this end, a multiplex RT-qPCR assay was developed for quick differentiation of FMDV and SVA (74). Multiplex assays are particularly vital for those countries that have multiple endemic viruses that cause vesicular disease in swine, because the ability to track different viruses will be critical to understanding viral epidemiology to develop control and prevention plans. Of equal importance to differentiation is the speed at which the diagnosis can be made. In countries free of FMDV, there is a halt to swine movement when a vesicle is observed until FMDV has been ruled out. Pen side testing allows quicker results and could contribute to faster continuity of animal movements. To this end, a field-deployable RT-insulated isothermal PCR (RT-iiPCR) has been developed that can detect SVA in the field (147). Unfortunately, this test can rule in/out SVA, but it does not provide information about FMDV status.

For countries with FMD negative status, identifying FMD would have severe economic ramifications including production loss, trade restrictions, control measures, and the cost of regaining FMD-free status (148). Due to the significant consequences involved with a FMDV positive diagnosis, testing for FMDV is highly regulated. In the United States, when a vesicle is observed in swine, a FAD investigation is instigated. Trained personnel collect a set of standard samples in duplicate to be sent to both the Foreign Animal Disease Diagnostic Lab (FADDL) and a National Animal Health Laboratory Network (NAHLN) lab to rule out FMDV. Therefore, even though pen side diagnostic tests for FMDV have been produced (RT-iiPCR and lateral flow device), governments may be reluctant to approve these platforms, due to ramifications of false positive/negative results (149-151). For example, a false negative result could lead to the

movement of positive animals and contribute to the spread of FMDV, which is considered one of the most highly contagious diseases of animals (152).

Not only is it important to be able to detect the virus in swine via PCR, it is equally important to be able to measure the antibody response to infection. As opposed to PCR, serology can provide information on SVA exposure over time. SVA VP1 and VP2 recombinant protein indirect ELISAs have been developed by diagnostic labs to identify SVA antibodies in swine, which are more rapid and convenient for diagnostic labs versus assays that involve cell culture, like virus neutralization (VN) assays and indirect IFA (79, 153). One group found antibody responses to VP2 were higher than VP1 and VP3 and had higher binding affinity in the ELISA, which correlates with data that VP2-specific IgG antibodies were shown to last the longest in experimentally challenged pigs (128, 153). Indirect ELISAs can have high-cross reactivity, so a competitive ELISA has also been developed (42, 154). Although the scalability of ELISA assays are favored by diagnostic labs, VN and IFA assays are excellent confirmatory assays with high sensitivity and specificity and often used in research settings (72, 154). Recently, a eGFP-tagged recombinant SVA has been developed to facilitate reading VN assays (155).

Both in situ hybridization (ISH) and IHC assays have been developed to detect SVA within tissues (134, 136). IHC assays detect viral antigen and require antibodies against the virus of interest, which in the case of SVA can be difficult to obtain commercially. In contrast, for RNAScope (ISH), a probe is ordered to a target genomic region of interest for virus detection in tissues (72, 134). Although it is rarely used for clinical diagnosis, electron microscopy has also been utilized to demonstrate particles with picornavirus morphology in animals infected with SVA (135).

Prevention/Control

Disinfectants have shown differing levels of success at inactivating SVA on different surfaces at various temperatures. In one study, bleach (sodium hypochlorite) at a 1:20 dilution was most effective at inactivating the virus, with a quaternary ammonium disinfectant demonstrating intermediate success depending on surface and temperature, and a phenolic disinfectant performing the worst (156). Accelerated hydrogen peroxide at 1:20 for 10 minutes was also an effective disinfectant against SVA, as well as FMDV and SVDV (157). Ultraviolet-C (UV-C, 254 nm wavelength) can also be used for an inactivation method, though it may be best suited as a redundant biosecurity measure because it was seen to be less effective with non-enveloped viruses and required greater than 3,000 J/L for viral inactivation of SVA (158).

Trypsin was suspected to be the source of contamination when SVA was discovered as a cell culture contaminant, due to evidence of swine being the natural host for SVA and since porcine trypsin is used commonly in cell culture work. Some swine vaccines are grown in cell culture, thus raising concern for SVA contamination during the vaccine manufacturing process. Vaccine distribution nationally and around the globe could serve as a route for the dissemination of SVA. Two lots of trypsin that had received 25-40 kGy of gamma-irradiation tested PCR positive and also virus isolation positive indicating live virus (159). Of note, after the trypsin samples received a second round of gamma-irradiation, SVA was inactivated. Thus, animal biologic manufacturers using porcine trypsin should add SVA to their exogenous agent testing to ensure that SVA is not inadvertently being spread through swine biologics.

Multiple vaccine platforms have been evaluated for efficacy against SVA challenge. A whole-virus inactivated vaccine made from a Chinese SVA isolate mixed with an adjuvant given in one dose provided protection against homologous challenge by preventing the development of clinical signs and viremia (160). Also, a recombinant SVA strain used as a live attenuated

vaccine given in a single dose induced a robust antibody response; and after challenge with SVA, animals did not develop any clinical disease, had reduced viremia, and had reduced viral shedding compared to non-vaccinated animals (161). Interestingly, an inactivated vaccine tested in the same study did not produce detectable neutralizing antibodies until after a second dose was given; and after challenge, the inactivated SVA vaccine did not protect against the development of vesicular disease (161). Inactivated virus vaccines do not contain live virus, so they are safer than a live attenuated virus vaccine that replicates and could revert to a virulent form. On the other hand, since live attenuated viruses replicate in the animal, they may generate a more robust humoral and cell mediated immune response than inactivated vaccines.

Recently, a virus-like particles (VLPs) vaccine for SVA has been tested in swine against SVA challenge. VLPs consist of viral structural proteins that spontaneously self-assemble into particles antigenically indistinguishable from the native virus (162). An advantage of VLPs is they present viral antigens in a more authentic conformation compared to typical subunit vaccines with recombinant proteins (163). Pigs vaccinated with SVA VLPs and challenged with a 2017 Chinese isolate did not develop clinical disease or viremia. This study also showed similar efficacy of a one-dose inactivated virus vaccine. Having an effective commercial vaccine for SVA could reduce the occurrence of SVA-related vesicular disease, thus reducing the economic burden of FADIs in FMD-free countries and viral load of SVA in endemic regions.

Swine Vesicular Diseases

There are currently five known viruses that cause clinically similar vesicular disease in swine: FMDV, SVDV, vesicular stomatitis virus (VSV), vesicular exanthema of swine virus (VESV), and SVA. Table 1 compares the etiology, epidemiology, and pathogenesis of these viruses. In addition to viruses, there has been a report of vesicular disease in white pigs due to ingestion of phototoxic compounds from celery and parsnips and cases of IVD where no

causative agent can be determined (164, 165). Since vesicular lesions appear clinically identical, diagnostics must be performed to determine the causative agent or rule out known causes. There is a multiplex PCR assay that can simultaneously detect VSV, SVDV, and FMDV (166). With the emergence of SVA, new multiplex assays will need to be developed (74). Depending on the status for each of the viruses in a country, one or more of these viruses can be reportable either nationally or internationally to the OIE.

FMDV is a OIE notifiable disease and can have severe consequences for the agriculture industry, international trade, and the economy. FMDV infects swine, but also other cloven-hoofed animals including cattle, sheep, goats, and wildlife (152). The FMDV outbreak in the United Kingdom in 2002 was estimated to cost around 10 billion dollars (167). Not only can it be economically devastating for FMDV-free countries experiencing an outbreak, but also for those countries where FMDV is endemic with estimates of production losses and vaccination costs between 6.5 and 21 billion dollars (168). FMDV is currently endemic in many countries in Asia, Africa, and the Middle East (169). It is critical to investigate any vesicular lesions in swine outside of these countries to rule out the presence of FMDV. FMDV is considered one of the most contagious diseases of livestock, so any delay in identification of the virus could lead to greater spread of the virus in a naïve population (170).

There are two distinct serotypes of VSV, Indiana (VSIV) and New Jersey (VSNJ) with VSNJ being responsible for most cases reported in the Americas (171). In experimental studies with swine VSNJ isolates were more virulent than VSIV (172). Outbreaks in livestock, mostly equines, in the US typically occur every 8-10 years in the west, but recently they have been occurring more frequently (173). The last reported incident of VS in swine in the US was 1968 (174). Virus has been isolated from biting and non-biting insects and they are believed to play a

major role in mechanical transmission (175). In addition, VSV is zoonotic and humans that have had contact with the virus have been infected (176).

SVDV has been considered endemic in Southern Italy and occasional outbreaks have occurred in other regions in Eastern and Western Europe and Asia (177, 178). As with SVA, subclinical infections of SVDV can be common (179). SVDV is very resistant to environmental factors such as freezing and dehydration, as well as common disinfectants (180). Interestingly, SVDV evolved from the human coxsackie B virus serotype 5 to make the species jump from humans to swine (181). Pigs challenged with coxsackie B virus did not develop clinical disease but did have a transient neutralizing response (182). SVDV along with VSV used to be OIE List A notifiable diseases but were de-listed in 2014 (183).

VESV was first discovered in 1932 in California and sporadic cases were recognized in swine fed raw garbage (184). In 1952 the virus spread to swine producing regions outside of California and was eradicated in 1959, which was thought to be associated with the enforcement of laws to prevent the feeding of garbage to swine (185). In 1972, San Miguel sea lion virus (SMSV) was isolated from California sea lions that developed fluid filled vesicles on non-haired portions of the flippers and is indistinguishable from VESV (186, 187). SMSV strains have subsequently been isolated from other marine mammals, fish, and flukes (188). Swine inoculated with various serotypes of SMSV have developed vesicular disease (189, 190).

As SVA spreads around the globe it will continue to present challenges due to its clinical similarity with FMDV. Improving knowledge about the epidemiology, viral evolution, and pathogenesis of SVA may help focus efforts directed at controlling the spread of SVA and future elimination efforts.

Table 2.1: Comparison of swine vesicular disease viruses.

	Foot-and-mouth disease virus (FMDV)	**Senecavirus A (SVA)**	**Vesicular stomatitis (VS)**	**Swine vesicular disease virus (SVDV)**	**Vesicular exanthema of swine virus (VESV)**
Etiology	Genus: Aphthovirus Family: Picornaviridae	Genus: Senecavirus Family: Picornaviridae	Genus: Vesiculovirus Family: Rhabdoviridae	Genus: Enterovirus Family: Picornaviridae	Genus: Vesivirus Family: Caliciviridae
Epidemiology	**Distribution**: Endemic in Asia, Africa, Middle East and parts of South America -US free since 1929 -The most contagious disease of animals **Transmission**: Aerosols, orally, direct or indirect contact, infectious meat **Hosts**: Cloven footed wild and domestic animals **Prevention**: Vaccine available but can be little cross protection between serotypes	**Distribution**: North America, Brazil, Colombia, China, Thailand, and Vietnam have confirmed cases - Found in swine samples since 1988 in the US **Transmission**: fecal-oral, direct or indirect contact **Hosts**: Pigs **Prevention**: Both an inactivated and a modified live vaccine have been tested in pigs and shown to be effective	**Distribution**: North and Central America and northern parts of South America -First recognized in swine in 1943 -Epidemics usually occur every 10 years **Transmission**: Insect vectors (flies) and direct contact. -Likely other unknown vectors **Hosts**: Pigs, horses, mules, donkeys, cattle, sheep, goats, deer, raccoons, and rodents **Prevention**: Vaccines have not been used for swine	**Distribution**: Europe and Asia -Porcine variant of human coxsackivirus B5 -First recognized in Italy in 1966 **Transmission**: infectious meat, fecal-oral, direct contact **Hosts**: Swine **Prevention**: No commercial vaccine and vaccination has never been undertaken	**Distribution**: United States -Eradicated in 1956 -San Miguel sea lion virus (SMSL) discovered in 1973 resulted in vesicles in sea lions and is indistinguishable -SMSL given to swine can produce the same disease as VESV **Transmission**: uncooked garbage with infectious meat from swine or fish, direct or indirect contact **Hosts**: Swine **Prevention**: No vaccine available

Table 2.1: Continued					
	Foot-and-mouth disease virus (FMDV)	**Senecavirus A (SVA)**	**Vesicular stomatitis (VS)**	**Swine vesicular disease virus (SVDV)**	**Vesicular exanthema of swine virus (VESV)**
Pathogenesis	**Incubation**: Ingestion 1-3d, exposure 3-5d -Primary site of replication in the tonsils with secondary in the skin -Carrier state in cattle, sheep and goats, but not swine -Swine considered amplifier hosts	**Incubation**: experimental 1-3d -Hypothesized to replicate in tonsils then spread via blood to other sites (skin) for further replication -Persistent infection has been shown in swine	**Incubation**: 3-5d (can be up to 21d) -Lesions first develop in stratum spinosum with vesicle formation, ulceration, and healing -Non-detectable viremia and no evidence of a carrier state	**Incubation**: Ingestion 2-3d, exposure 2-7d -Thought to replicate at an initial site and then travel through lymphatics to the blood stream for travel to sites such as the coronary band -Carrier animals rare -Reports of virulent and avirulent strains	**Incubation**: 18-72h -Low grade viremia, virus replicates in the basal layer of the epidermis and results in vesicle formation, but basal layer remains intact
Clinical signs for ALL	-Fever, lameness, vesicles on the coronary bands, intradigitally, and snouts that progress to erosions. Clinical signs often resolve in a couple weeks.				
Additional clinical signs	-Lesions in oral mucosa, salivation -Cardiomyopathy and possibly death in young animals	-Hoof wall hemorrhages observed in some animals post clinical signs	-Vesicles on the lips and snout may cause excessive salivation -Vulva, scrotum, and teats can have lesions -Humans can get fever, headache, and oral vesicles	-Salivation, neurologic signs, more severe in young animals	-Mild meningitis in one lab worker infected

* Information gathered from the Swine Disease Manual (191) and the National Veterinary Accreditation Program (192).

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CHAPTER 3. DEXAMETHASONE TREATMENT DID NOT EXACERBATE SENECA VALLEY VIRUS INFECTION IN NURSERY-AGE PIGS

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Modified from a manuscript published in *BMC Veterinary Research*

Abstract

Background: Senecavirus A, commonly known as Seneca Valley virus (SVV), is a picornavirus that has been infrequently associated with porcine idiopathic vesicular disease (PIVD). In late 2014 there were multiple PIVD outbreaks in several states in Brazil and samples from those cases tested positive for SVV. Beginning in July of 2015, multiple cases of PIVD were reported in the United States in which a genetically similar SVV was also detected. These

events suggested SVV could induce vesicular disease, which was recently demonstrated with contemporary US isolates that produced mild disease in pigs. It was hypothesized that stressful conditions may exacerbate the expression of clinical disease and the following experiment was performed. Two groups of 9-week-old pigs were given an intranasal SVV challenge with one group receiving an immunosuppressive dose of dexamethasone prior to challenge. After challenge animals were observed for the development of clinical signs and serum and swabs were collected to study viral shedding and antibody production. In addition, pigs were euthanized 2, 4, 6, 8, and 12 days post inoculation (dpi) to demonstrate tissue distribution of virus during acute infection.

Results: Vesicular disease was experimentally induced in both groups with the duration and magnitude of clinical signs similar between groups. During acute infection [0-14 days post infection (dpi)], SVV was detected by PCR in serum, nasal swabs, rectal swabs, various tissues, and in swabs from ruptured vesicles. From 15 to 30 dpi, virus was less consistently detected in nasal and rectal swabs, and absent from most serum samples. Virus neutralizing antibody was detected by 5 dpi and lasted until the end of the study.

Conclusion: Treatment with an immunosuppressive dose of dexamethasone did not drastically alter the clinical disease course of SVV in experimentally infected nursery aged swine. A greater understanding of SVV pathogenesis and factors that could exacerbate disease can help the swine industry with control and prevention strategies directed against this virus.

Background

Vesicular disease in swine is recognized by the development of vesicles on the feet, snout, and less frequently in the oral cavity (1, 2). Nonspecific signs include fever, lethargy, anorexia, and lameness. Known viral causes of swine vesicular disease are vesicular stomatitis

virus, swine vesicular disease virus, vesicular exanthema of swine virus, and importantly, foot-and-mouth disease virus (FMDV). FMDV causes one of the most highly contagious diseases of livestock that can result in devastating economic losses to the agricultural industry and disruption of the human food supply (3, 4). Since vesicular diseases of swine are indistinguishable in the field, each case must be treated as if it was an FMDV infection, which triggers a significant response in countries where FMDV is not endemic. Although rare, swine vesicular diseases have occurred in countries without known vesicular viruses or the known causes have been ruled out, resulting in the diagnosis of porcine idiopathic vesicular disease (PIVD).

Senecavirus A, commonly known as Seneca Valley virus (SVV), is a non-enveloped, single-stranded RNA virus in the family *Picornaviridae* first identified as a tissue culture contaminant in 2002 (5). A retrospective analysis confirmed that since the late 1980s, SVV has been sporadically isolated from swine samples in the United States (US) (6), and detected by PCR in more recent cases of PIVD (7, 8). Although early attempts to induce vesicular disease with field SVV isolates were unsuccessful (6, 9-11), it was presumed that SVV was the causative agent.

In late 2014, outbreaks of vesicular disease in finishing swine, and in sow farms with concurrent reports of increased neonatal mortality occurred in multiple states in Brazil (12, 13). As part of the diagnostic investigations ruling out known causes of vesicular disease in swine, SVV was identified by PCR and virus isolation in multiple samples from affected animals. In July 2015, similar outbreaks began in the US and SVV was also detected in those cases (14-17). Collectively, the association of SVV with vesicular disease in Brazil and the US provided strong support for SVV as the causal agent. This was confirmed with the fulfillment of Koch's postulates in 9-week old pigs using a 2015 SVV isolate from the US (18). Since that report,

vesicular disease was also experimentally reproduced with SVV infection in nursery pigs (19) as well as in finishing-aged swine (20). Although SVV was rarely detected in North America prior to the 2014/2015 unprecedented emergence of PIVD in Brazil and the United States, it has been detected many times since then in the respective countries as well as recent novel case reports in Canada (21), China (22-24), Thailand (25), and Colombia (26). Interestingly, viruses from these recent outbreaks are genetically similar sharing > 94% nucleotide identity at the full-length genomic level.

In an early PIVD report there was speculation that stressful events in the field may predispose pigs to SVV clinical disease; e.g., after transportation to slaughter (8). Similar observations in the 2014/2015 SVV cases supported this assumption which led to the original experiment using an immunosuppressive model to test the hypothesis that administration of a synthetic glucocorticoid would exacerbate the SVV infection in swine. Surprisingly, both non-dexamethasone treated pigs as well as dexamethasone treated pigs developed vesicular disease of comparable severity. The acute phase of the vesicular disease in the non-dexamethasone SVV-challenged pigs was previously reported (18). This manuscript describes the kinetics of the SVV infection and the comparison between the dexamethasone and non-dexamethasone treated pigs.

Results

Clinical and microscopic observations

All pigs were free from signs of vesicular disease prior to challenge, and all control pigs appeared normal throughout the experiment. One pig in the Dex-SVV group became anorexic at 2 dpi and was removed from the experiment because it was not competitive in a group environment. The pig's health continued to deteriorate, and it died 2 days post removal from the group. Although no definitive cause of death was determined, it is believed SVV did not

contribute to the illness and death since the only clinical signs recognized in the other pigs was transient lameness.

A mild transient lameness was recognized in 2-3 pigs from both the Dex-SVV and SVV groups on 2 and 3 dpi. No gross abnormalities in behavior or appearance were observed in pigs euthanized on 2, 4, 8, and 12 dpi for necropsy.

The acute lesions for the SVV pigs were previously described (18). The lesions that developed in the Dex-SVV group were indistinguishable from the SVV group and are briefly described below. At the 4 dpi daily observation, cutaneous lesions were detected in 8/11 Dex-SVV pigs (72.7%) and 7/16 SVV pigs (43.8%). Cutaneous lesions consisted of small vesicles (about 3mm x 3mm) and/or erosions first noticed at 4 dpi in the interdigital spaces and coronary bands of one or more feet. At 5 dpi, all Dex-SVV pigs were observed with vesicular lesions and 14/15 SVV pigs had at least one lesion. Lesions were recognized as small, pale or blanched areas of swelling on the coronary band that would grow in size, thicken and become raised (Figure 1). Usually, the skin would wear away leaving an erosion or ulcer that could coalesce with adjacent lesions. Snout lesions, when present, were mostly recognized as an elliptical erosion (3mm x 5mm) that was on the dorsal ridge of the snout which quickly healed. No new coronary band lesions were recognized after 6 dpi at which time the lesions began to heal.

Figure 3.1: Vesicular lesions from 9-week-old swine. A) Ruptured vesicle in the interdigital space. B) Intact vesicle on the lateral coronary band.

Microscopic examination of coronary band tissue sections from the pigs euthanized revealed lesions in the 4 and 6 dpi pigs. Extensive areas of epidermis, predominantly stratum spinosum, was affected and effaced by multifocal to coalescing vesicles, containing fibrin, edema, necrotic debris and infiltration of lymphocytes and plasma cells (Figure 2). Occasionally, these vesicles progressed to pustules, which were characterized by degenerate neutrophils admixed with variable amounts of fibrin, cellular and karyorrhectic debris. A few of these inflammatory cells were multifocally observed in the superficial dermis (beneath the affected epidermis).

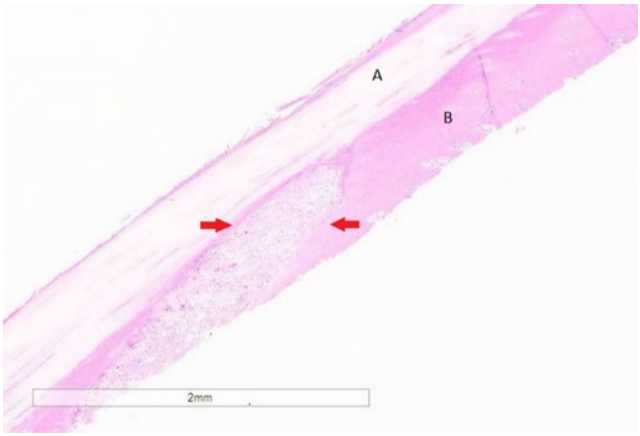

Figure 3.2: Microscopic lesions from the coronary band. Cut section of the epidermis with the (A) stratum corneum and (B) stratum spinosum. Red arrows point to an oval shaped vesicle disrupting the stratum spinosum, which contains small numbers of neutrophils, lymphocytes, varying amounts of fibrin, edema admixed with necrotic and cellular debris.

SVV RNA detection

Selected samples from control animals all tested negative for SVV RNA by PCR. In serum samples of both Dex-SVV (n=11) and SVV (n=12) groups SVV RNA was detected in pigs as early as 1 dpi (Figure 3). For the Dex-SVV group, peak viremia occurred at 5 dpi with a mean value of 5.7 $x10^4$ genomic copies per microliter (GC/µL) of serum. Peak of viremia was observed at 3 dpi in the SVV group with a mean value of 3.1 $x10^5$ GC/µL of serum. Though different peaks were observed, no statistically significant differences were detected in the

magnitude of viremia between Dex-SVV and SVV groups. Mean SVV RNA concentration in serum in both groups decreased over time with minimal amounts detected at 15 (0.6 GC/µL) and 22 dpi (0.3 GC/µL).

In nasal swabs, SVV RNA was detected in one or more pigs at all time-points of the study, with a peak mean value of 2.0 $x10^5$ GC/µL in the Dex-SVV group and 8.8 $x10^4$ GC/µL in the SVV group at 3 dpi (Figure 3). By 30 dpi, GCs were found at low levels in all animals of the SVV group and only three animals were PCR positive in the Dex-SVV group. There was no statistically significant difference in GC/µL levels between the Dex-SVV and SVV group.

In rectal swabs, SVV RNA levels were approximately 2 to 3 logs lower than those in nasal swabs (Figure 3). An observable peak occurred at 3 dpi for the Dex-SVV group (9.5 $x10^2$ GC/µL) and at 5 dpi for the SVV group (2.6 $x10^2$ GC/µL) with statistically significant differences between the two groups detected at 3 and 5 dpi ($p < 0.05$). In addition, both experimental groups had a transient increase in SVV RNA levels from 11 dpi to 15 dpi.

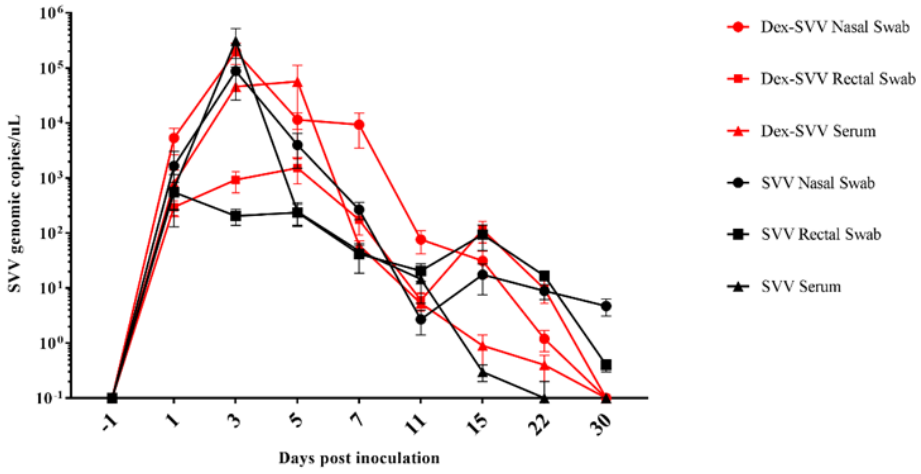

Figure 3.3: SVV infection dynamics. Virus shedding in nasal and rectal swabs and viremia levels detected by RT-qPCR. Quantity of viral RNA is expressed as genomic copies per microliter. Error bars represent the standard error of the mean. Red data lines represent the Dex-SVV group and black data lines represent the SVV group. Nasal swabs are designated with a circle, rectal swabs with a square, and serum with triangles. No viral shedding or viremia was detected in control animals.

SVV RNA was detected in almost every tissue tested in each pig necropsied at 2, 4, 6, 8, and 12 dpi (Figure 4). In general, the lowest RNA levels were detected in pig 234 (2 dpi) with peak RNA levels detected in pig 235 (4pi) followed by a reduction over time for pigs 236 (6 dpi), 237 (8 dpi), and 238 (12 dpi), respectively. SVV RNA was found at much higher concentrations in the tonsil (7.83 x10^5 GC/µL at 4 dpi) and inguinal lymph nodes (6.55 x10^5 GC/µL at 6 dpi) compared to the other tissues.

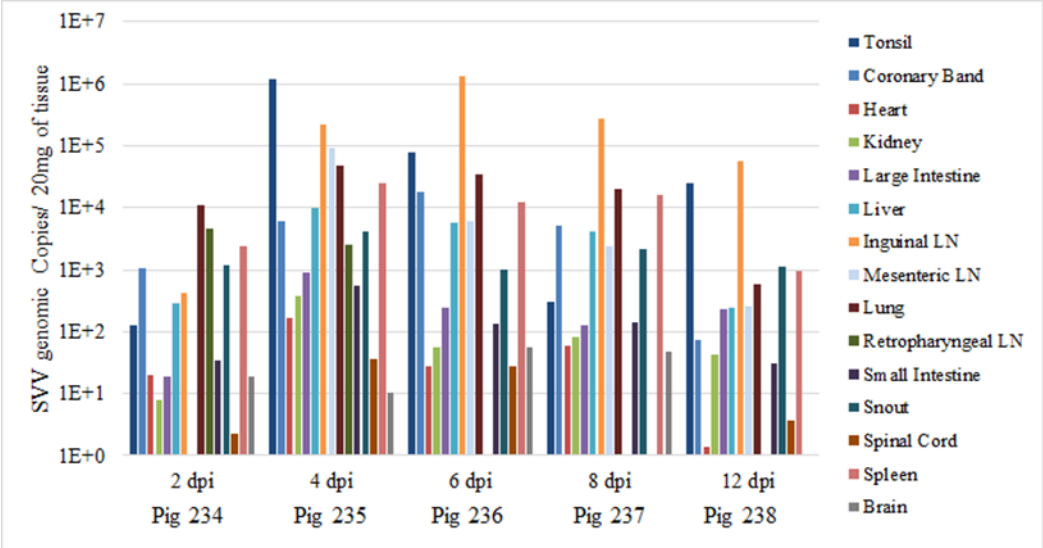

Figure 3.4: Tissue distribution of SVV. Viral load present in selected tissues from necropsied pigs on 2, 4, 6, 8, and 12 dpi respectively. Quantity of viral RNA was determined by RT-qPCR and expressed as genomic copies per 20 mg of tissue.

Serological responses

All 0 dpi sera samples were negative for antibodies against SVV by IFA and VN assays. All challenged pigs developed IgG IFA antibodies against SVV by 11 dpi with titers ranging from 1:320 to 1:640. Pigs from both groups had a similar IgG response throughout the study with most having a titer of 1:1280 on 30 dpi (Figure 5). All but 2 pigs in the Dex-SVV group developed measurable neutralizing antibodies by 5 dpi. VN titers peaked in both groups at 7 dpi.

Again, the neutralizing antibody response was similar for both the Dex-SVV and SVV groups over the course of the experiment as shown in Figure 5. Control pig sera had VN titers ≤ 1:4.

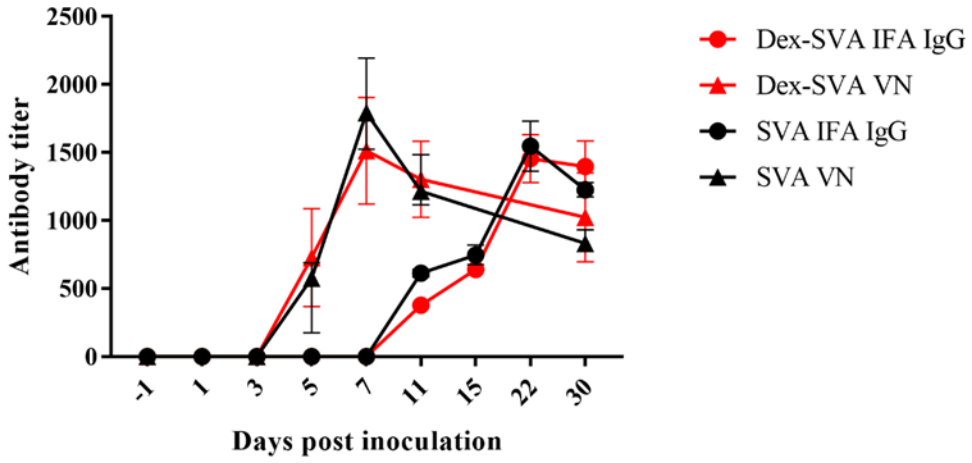

Figure 3.5: Antibody response to SVV infection. Indirect immunofluorescence assay measured IgG antibody response to SVV infection. Virus neutralization assay measured neutralizing antibody response to SVV. Red data lines represent the Dex-SVV group and black data lines represent the SVV group. IFA titers are depicted with circles while VN titers are designated with triangles. Error bars represent the standard error of the mean.

Discussion

Our hypothesis for this study was that stress induced through an immunosuppressive dose of dexamethasone would exacerbate clinical disease in SVV challenged pigs compared to those not treated with dexamethasone. This experimental question was derived from failed attempts prior to 2016 to reproduce vesicular disease with SVV, and the observations that PIVD cases were associated with times of stress such as transportation of finishing pigs to market (8) and congregations of show pigs. In this study, SVV infection induced prolific vesicular lesions in both the SVV and Dex-SVV treatment groups. The onset, character, and duration of the lesions were similar between groups indicating the dexamethasone treatment did not dramatically alter the clinical disease of pigs after SVV challenge. More pigs developed vesicular lesions earlier in

the Dex-SVV group, but by 5 dpi all but one pig in this experiment developed clinical signs. For the purposes of this discussion, both groups will be discussed as one unless noted otherwise.

Development of vesicular disease in both challenge groups was surprising since experimental infections reported before 2016 were unable to induce clinical disease (6, 9-11). Vesicular disease reproduced in this study was compatible with early field reports of SVV-related PIVD in finishing-aged pigs and sows (12, 13, 17), and similar to recent reports of experimentally induced vesicular lesions with US wild-type virus in 3-week-old pigs (19) and 55 kg pigs (20). Collectively, this research may provide insight into the kinetics of SVV-associated vesicular disease that can provide an evidence for recommendations for diagnostic sampling and possible control strategies.

In this study, lesions were first noted in the interdigital spaces when pigs were observed at 4 dpi. It is likely the onset of foot lesions began at an earlier time-point, given that by 4 dpi some pigs already had both intact and ruptured vesicles on their feet. The incidence of foot lesions peaked about 5-6 dpi and most lesions had resolved in about a week after onset of clinical signs. Our timeline of vesicular lesion development is similar to that reported by Joshi et al. in finishing pigs experimentally inoculated with SVV where lesions were first observed at 4 dpi (20). In contrast to onset and incidence of coronary band lesions, snout lesions observed in this study were first recognized around 7 dpi in just a few pigs as one or two small elliptical plaques on the dorsal ridge of the snout. In comparison, a higher incidence of snout lesions was reported in older (20) and nursery age pigs (19).

Viremia and viral shedding were measured by RT-PCR to gain a better understanding of SVV infection kinetics. PCR results revealed a rapid onset of viremia with many pigs testing positive for SVV in serum by 1 dpi. Peak RNA concentration in sera occurred at 3-5 dpi

followed by a rapid decrease becoming almost undetectable by 15 dpi. Viral shedding also had a rapid onset with both nasal and fecal swabs testing positive for SVV at 1 dpi. The Dex-SVV group had statistically higher levels of SVV detected by PCR at 3 and 5 dpi in the rectal swabs. Virus was detected longer in nasal swabs than fecal swabs, especially in the SVV group. By 30 dpi most pigs in either group were SVV-negative on rectal swab, but some of the SVV group were still nasal swab positive for viral RNA, though there was no statistical difference between groups. A similar shedding pattern was observed by Joshi et al. who reported that oral, nasal, and fecal swabs became PCR negative by 28 dpi in finishing pigs (20). Oral swabs were not collected in the present study, but others have reported a greater amount of virus detected and longer shedding compared to fecal or nasal swabs (19, 20).

In the pigs euthanized from 2 – 12 dpi, 4 dpi was the time point with highest detection of SVV nucleic acids in tissues. In general, the tissues with the greatest SVV RNA presence were coronary band epithelia, tonsils, inguinal and mesenteric lymph nodes, lung, liver, and spleen. In addition, most tissues had higher SVV RNA concentrations than serum, which suggests that viral concentrations in the tissues is not solely due to blood contamination. A similar tissue distribution was reported in mid-finishing swine during acute infection in addition to lymph nodes, spleen, intestine, kidneys, and tonsils testing positive by PCR 38 days after challenge (20). Similarly, diagnostic investigations conducted in sow herds affected by vesicular disease and neonatal mortality in the US and in Brazil described detection of SVV nucleic acids in most tissues of piglets including the small intestine, tonsil, lung, heart, liver, spleen, kidney, myocardium and cerebellum (27, 28). Previous field case reports have documented detection of SVV in scrapings of ruptured vesicles and ulcerative lesions by PCR in sows and finishing pigs

(7, 12, 13, 28). Based on PCR results in our study, vesicular fluid/swabs had the highest concentration of virus compared to other samples.

The onset of the humoral immune response was similar for both groups with antibody titers first being detected at 5 dpi by VN assay and 11 dpi by IFA assay. The onset and peak of antibody titers was more rapid for neutralizing antibody response compared to IgG measured by IFA assay. Similarly, Joshi et al. detected VN antibody as early as 5 dpi, which was also described by Yang et al., and SVV-specific IgG antibodies by 10 dpi (11, 20). Chen et al. reported neutralizing antibody response as early as 3 dpi in weaned pigs (19). Aforementioned studies have credited the early neutralizing antibody response for the quick decline in viremia reported in various aged swine. The duration of a SVV humoral immune response is unknown though unpublished work has demonstrated sterilizing immunity in gilts challenged five months after initial exposure to wild type SVV (Buckley et al., unpublished).

Seneca Valley virus is in the same virus family as FMDV and shares many structural characteristics as well as a similar clinical presentation. Vesicular lesions can develop within 24-48 hours of FMDV challenge (2, 3, 29), and are most commonly found on the coronary bands (30). FMDV can also be detected in serum as soon as 24 hours after exposure followed by gradual decline over a couple of weeks (31). Antibodies against FMDV have been detected by ELISA around 7 dpi (IgM) and 14 dpi (IgG, IgA) in swine either inoculated or in direct contact (32). Although vesicular lesions contain the highest concentration of FMDV, virus replication does occur in other tissues, e.g., in the tonsil (31). This study and others have consistently found high concentrations of SVV nucleic acids present in the tonsil not only acutely, but also in convalescent swine (15, 20), which may provide evidence for the tonsil being a primary site of viral replication. The kinetics of the SVV infection are similar to FMDV; however, it is unknown

if SVV is as infectious as FMDV. Replication and shedding of SVV from the tonsil into the oropharyngeal cavity probably plays an important role in producing SVV-positive oral secretions, and may contribute to contamination of the nasal passages and the finding of SVV-positive nasal swabs. However, the possible replication of SVV in nasal mucosa has not been ruled out.

Unpublished observations from our laboratory support recent field reports describing environmental contamination with SVV nucleic acid (33), which combined with normal pig behavior such as rooting and inquisitive chewing, could cause false-positives in oral fluid samples. The extended presence of SVV in tonsil tissue may help explain the apparent longer shedding patterns reported for oral fluids and nasal swabs when compared to fecal swabs (19, 20). Further study will be required under experimental conditions to characterize the duration of shedding of infectious virus from the oral cavity.

Results from Chen et al., Joshi et al., and the present study can certainly be used for diagnostic recommendations for nursery to mid-finishing pigs that will be presented dogmatically for simplicity. Viral RNA can be detected in serum from at least 2-7 days, in feces (rectal swabs) from at least 2-15 days, in nasal swabs from at least 2-15 days, and in oral fluids from at least 2-21 days post infection. During the acute infection viral RNA can be detected in many tissues for at least 2-12 days post infection and can also serve as potential diagnostic samples. Finally, swabs of vesicular lesions may serve as the best sample to collect during clinical disease to reliably detect the presence of SVV due to the large quantities of virus found in that location. Lastly, it is expected that sows infected with SVV will have similar infection kinetics to nursery and mid-finishing swine and sample recommendation listed above would also pertain to mature animals.

Compared to other species, swine are relatively resistant to glucocorticoids (34, 35); however, their use has induced recrudescence of latent pseudorabies virus in swine indicating glucocorticoids can alter or suppress a pig's immune system (36). Dexamethasone administration in this study was based on previous studies that successfully induced recrudescence of wild-type and attenuated PRV vaccine (36), and presumably this dosing schedule would be a substitute for stress that would alter the immune system allowing for enhanced vesicular disease. The Dex-SVV group had more pigs observed with vesicular lesions on 4 dpi, but by 5 dpi all except 1 pig was effected in both groups. In addition, there were higher concentrations of SVV detected in rectal swabs on 3 and 5 dpi compared to the SVV group. It is unknown if a higher glucocorticoid dose might have altered the disease course more significantly, but such efforts may not be needed with contemporary SVV isolates based on this experiment and the previous successful reports (19, 20)

The relative ease in which vesicular disease was produced in both groups of pigs in this study, and in the experiments of Chen et al. and Joshi, et al. provides a basis for a variety of questions and assumptions about contemporary SVV isolates, and why attempts to reproduce vesicular disease prior to 2016 were unsuccessful. First, the genetic similarity of current SVV isolates from Brazil, U.S., Canada, China, Thailand, and Colombia suggests a recent common ancestor which might explain the timing of newly recognized outbreaks across separate continents. Second, there may be a mutation in SVV that increased the likelihood that contemporary viruses are more likely to induce vesicular disease than viruses isolated before 2014. If either of these factors played a role in the emergence of the mini SVV epidemics, then there must be some explanation for the relatively recent distribution of the common virus among continents. However, an increased awareness of potential SVV-related vesicular disease may be

contributing to the current observations. For example, the increase in reports of clinical disease associated with SVV isolates in China (24) and Thailand (25) that are more distantly related genetically to the contemporary Brazilian and US isolates suggests current clinical cases are not completely dependent on the emergence of a new lineage of virus. Third, there may be a novel cofactor(s) such as another infectious agent allowing disease to develop, which if true, must also have been transmitted to different continents. Fourth, there may be a genetic predisposition to clinical disease that is more prevalent now when compared to pre-2014 swine, and this trait has been slowly building in swine herds in countries reporting recent events. In addition, there may be an age-dependent effect on clinical presentation of disease which makes it more difficult to observe disease in young pigs compared to mature swine. The focal nature of vesicular disease demonstrates a discrete viral tropism for a cell type in a specific anatomical site, i.e., coronary band tissue. Such a condition might involve an increased density of this cell type in older swine, or this tissue may be more prevalent in one genetic line compared to another which would contribute to the presence of gross lesions. Lastly, there is the possibility that two or more of the above factors contributed to the emergence of the mini SVV epidemics.

Conclusions

This study adds to the growing body of knowledge about the pathogenesis of SVV in swine that has focused on understanding the acute infection (clinical disease, viremia, viral shedding, virus distribution in tissues, and immune response). This work has demonstrated the need to investigate the duration of immunity in convalescent swine and the spectrum of immunity against historical and contemporary strains to discern why an apparent change has occurred in the ecology of SVV in swine. The clinical description and kinetics from this study

can also help diagnosticians and veterinarians improve strategies to control this disease and differentiate it from other vesicular diseases in swine.

Methods

Cells and virus

A swine testicular (ST) cell line (ATCC® CRL-1746; American Type Culture Collection, Manassas, VA) was cultured at 37 °C and 5% CO2 in minimum essential medium (MEM, MilliporeSigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, AtlantaBio, Flowery Way, GA), 1x gentamicin, and 1x glutamax (Life Technologies, Carlsbad, CA). NCI-H1299 cells (ATCC® CRL-5803) were cultured under the same conditions in Dulbecco's modified Eagle's medium (DMEM, MilliporeSigma) supplemented with 10% FBS (AtlantaBio).

SVA15-41901SD was isolated on ST cells from samples collected from a barn of finishing pigs in South Dakota that developed vesicular lesions during the summer of 2015 (15). The second and third passage of the virus were combined to create a larger volume of working stock virus which was utilized for inoculum and laboratory assays. The stock virus had a titer of 4.75×10^7 plaque-forming units (PFU)/ml and was tested for purity by next-generation-sequencing that only detected SVA.

Animals and experimental design

Forty-nine conventionally-raised weaned pigs were purchased, randomly assigned ear tags and housed at the USDA-ARS-NADC campus in accordance with Institutional Animal Care and Use Committee guidelines (ACUP #2867) until 9 weeks-of-age at which time 29 pigs received SVV at a dose of 4.75×10^7 PFU/animal via intranasal inoculation. A LMA® MAD Nasal™ Intranasal Mucosal Atomization Device (Teleflex; Morrisville, NC) was used for delivery of about 2.5 mL of the atomized inoculum into each nostril for a total of 5 mL. Twelve

pigs (Dex-SVV group) based on sequential ear tag numbers were given an immunosuppressive treatment of dexamethasone (AgriLabs; St. Joseph, MO) intramuscularly in the neck for 5 days prior to challenge as follows: day 1, 2.3 mg/kg of body weight twice a day (BID); days 2-5, 1.1 mg/kg BID. Seventeen pigs (SVV group) did not receive any treatment before challenge. The remaining 20 pigs were unchallenged control pigs (Control group). Each group was housed in a separate animal-biosafety-level 2 room, with at least 17 square feet/pig and constant access to feed and water. One pig each from the SVV group was euthanized on 2, 4, 6, 8, and 12 days post inoculation (dpi). At the time of euthanasia, the animal was physically restrained for the intravenous administration of a barbiturate (Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI) following the manufacturer label dose (1 mL/4.54 kg). At the conclusion of the study the remaining pigs were also euthanized in the same manner.

Sample collection

Progression of clinical disease for each pig was assessed by daily observations for lameness and vesicular lesions. Antemortem sampling consisted of collection of whole blood in serum separation tubes (BD Vacutainer®, Franklin Lakes, NJ), and nasal and rectal swabs. Swabs (Puritan Medical Products, Guilford, ME) were collected and immersed in 3 ml of serum-free MEM (MilliporeSigma). Samples were collected on 0, 1, 3, 5, 7, 11, 15, 23, and 30 dpi from the SVV and Dex-SVV groups. Pigs from the control group were sampled at 0, 7, 15, and 29 dpi. When vesicles were observed, vesicular fluid collection was attempted via aspiration or swabs. Blood tubes were centrifuged to harvest the serum. Sera, vesicular fluids, and swab tubes were stored at -80 °C until time of testing.

In the pigs euthanized on 2, 4, 6, 8, and 12 dpi, all postmortem examinations were performed immediately after euthanasia, and tissue specimens collected included snout and coronary band epithelium, tonsil, lymph nodes (retropharyngeal, mesenteric, and/or inguinal),

lungs, heart, liver, spleen, kidney, small and large intestine, brain and spinal cord. For each anatomically defined specimen, one approximate 10 g of tissue was collected and placed into a self-sealing plastic bag on dry ice for transfer within 2 hours to a -80 °C freezer. Tissues were thawed and 20 mg of each sample resuspended in 5 mL 1x PBS (Thermo Scientific, Waltham, MA) in individual gentleMACS™ M tubes (Miltenyi Biotec, Auburn, CA). Tissue was dissociated using a gentleMACS™ Octo-Dissociator (Miltenyi Biotec, Germany) following the manufacturer's recommendations. After dissociation, tissue suspensions were aliquoted in 2 mL cryogenic vials (Corning, Corning, NY) and frozen at -80 °C until time of processing. For histology, skin sections from the coronary band region were collected in 10% neutral buffered formalin. Sections were fixed in formalin for 24 hours, routinely processed, paraffin-embedded, sectioned at 4-µm thickness, and stained with hematoxylin and eosin.

Seneca Valley virus-specific nucleic acid detection

Samples were extracted using the MagMAX™ Pathogen RNA/DNA kit (Life Technologies, Carlsbad, CA) following manufacturer's recommendations and a MagMAX™ Express instrument 24 (Life Technologies) using program AM1836 (Life Technologies). The viral RNA was eluted in 90 µL of elution buffer. Following extraction, 5 µL of the nucleic acid templates were added to 20 µL of the Path-ID™ Multiplex One-Step RT-PCR reaction master mix (Applied Biosystems, Foster City, CA) for fecal swabs or 20 µL AgPath-ID™ One step RT-PCR kit (Applied Biosystems) for nasal swabs, vesicle fluids, and sera. The primers and probe were designed to target the conserved region between the 5' untranslated region (5'UTR) and protein L containing nucleotides 602-710 of the SVA genome. The forward primer sequence was 5'-TGCCTTGGATACTGCCTGATAG-3', the reverse primer sequence was 5'-GGTGCCAGAGGCTGTATCG-3' and the probe sequence was 5'-CGACGGCCTAGTCG GTCGGT T-3'. The probe was labeled using 6-FAM™ at the 5' end, ZEN™ internal quencher,

and Iowa Black® quencher at the 3' end (Integrated DNA Technologies, Coralville, IA). Real-time RT-PCR was performed on an ABI 7500 Fast instrument (Life Technologies) run in standard mode with the following conditions: 1 cycle at 48 °C for 10 min, followed by 1 cycle at 95 °C for 10 min, and 40 cycles of 95 °C for 15 sec and 60 °C for 45 sec. SVA genome RNA copies were calculated based on a standard RNA transcript overlapping the target region.

Indirect immunofluorescence (IFA) assay

SVV-specific IgG antibody response to SVV challenge was evaluated by an IFA assay. Serum samples were serially diluted 1:2 up to (1:5120). Plates were previously prepared with SVV-infected NCI-H1299 cells overnight and fixed with cold 80% acetone, and stored at -20 °C. Plates were rehydrated with 200 µL of PBS and 50 µL of diluted serum were added to wells to incubate for 1 hour. Plates were washed 3 times and 1:50 diluted anti-swine IgG-FITC antibody (KPL, MD, USA) was added to incubate for 45 min. Again, plates were washed 3 times and wells were observed for fluorescence under a fluorescent microscope. The highest serum dilution with clear and specific staining was considered as the end point (i.e., IFA titer).

Virus neutralization (VN) assay

Serum samples were heat-inactivated at 56 °C for 30 min and serially diluted 1:4 (up to 1:4096) in MEM in a 96-well plate and repeated in quadruplicate. Each diluted serum was mixed with an equal volume of SVA15-41901SD (200 $TCID_{50}$) and incubated for 1 hour at 37 °C. One-hundred microliters of each virus-serum mixture was transferred to respective wells of 96-well plates of ST cells grown to a confluent monolayer and replenished with MEM supplemented with 2% FBS. Plates were read for CPE daily for 4 days. VN titers, based on CPE, were recorded as the highest dilution of serum at which the infectivity of SVA-41901SD was completely neutralized in 50% of the inoculated wells. A back titration of the virus was performed during each test.

Statistical analysis

Data analyses and graphic representations were performed by using Microsoft Office Excel 2010 and GraphPad Prism 7.03. Statistical analyses of the data were performed using a mixed linear model (SAS 9.4 for Windows XP, SAS Institute Inc., Cary, NC, USA) for repeated measures and a spatial spherical or autoregressive covariance structure was utilized. Linear combinations of the least squares means estimates for GC/uL were used in a priori contrasts after testing for either a significant ($P<0.05$) effect of treatment or a significant treatment by time interaction. Comparisons were made between treatment groups at each time-point using 5% level of significance ($P<0.05$) to assess statistical differences.

Ethics approval and consent to participate

This study was approved and performed in accordance with Institutional Animal Care and Use Committee guidelines (ACUP #2867) at the USDA ARS National Animal Disease Center. Animals were purchased from a USDA-registered vendor in Wisconsin for research purposes.

Acknowledgements

The authors thank Deb Adolphson and Sarah Anderson for technical assistance and Jason Huegel, Justin Miller, and Keiko Sampson for assistance with animal studies.

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CHAPTER 4. COMPARISON OF HISTORICAL AND CONTEMPORARY ISOLATES OF SENECAVIRUS A

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Modified from a manuscript under review in *Veterinary Microbiology*

Abstract

Senecavirus A (SVA) was discovered as a cell culture contaminant in 2002, and multiple attempts to experimentally reproduce disease were unsuccessful. Field reports of porcine idiopathic vesicular disease (PIVD) cases testing PCR positive for SVA in addition to outbreaks of PIVD in Brazil and the United States in 2015 suggested SVA was a causative agent, which has now been consistently demonstrated experimentally. Ease of experimental reproduction of disease with contemporary strains of SVA raised questions concerning the difficulty of reproducing vesicular disease with historical isolates. The following study was conducted to compare the pathogenicity of SVA between historical and contemporary isolates in growing pigs. Six groups of pigs (n=8) were intranasally inoculated with the following SVA isolates: SVV001/2002, CAN/2011, HI/2012, IA/2015, NC/2015, SD/2015. All isolates induced vesicular disease in at least half of the inoculated pigs from each group. All pigs replicated virus as

demonstrated by serum and/or swab samples positive for SVA by quantitative PCR. Pig sera tested by virus neutralization assay demonstrated cross-neutralizing antibodies against all viruses utilized in the study. Cross-neutralizing antibodies from pigs inoculated with historical isolates were lower than those pigs that were inoculated with contemporary isolates. Phylogenetic analysis revealed two clades with SVV001/2002 being in a separate clade compared to the other five isolates. Although differences in the infection kinetics and sequences of these six isolates were found, clinical presentation of vesicular disease was similar between both historical and contemporary isolates.

Introduction

Senecavirus A (SVA) is a non-enveloped, positive-sense, single-stranded RNA virus ~7.2 kb in length (1). SVA is the only member of the genus *Senecavirus* in the family *Picornaviridae* and shares closest sequence identity to the genomes of those viruses in the *Cardiovirus* genus (2). SVA has one large open reading frame (ORF) encoding a single polyprotein that is then cleaved by viral proteases into 12 mature proteins in the standard picornavirus L-4-3-4 layout (1). The icosahedral viral capsid consists of 60 protomers each containing the structural proteins VP1, VP2, VP3, and VP4 (located internally) with lengths of 265, 286, 241, and 74 amino acids (AA) respectively (3). The single ORF is flanked by a long 5'untranslated region (UTR) (666 AA) and short 3'UTR (71 AA) followed by a poly(A) tail (4).

SVA was first discovered as a cell culture contaminant at a lab in Maryland in 2002 and was named Seneca Valley virus-001 (SVV-001) (1). The source of contamination was speculated to be porcine trypsin or fetal bovine serum (5). Picornavirus-like viruses had been isolated from sporadic swine samples at the National Veterinary Services Laboratory (NVSL) beginning in 1988 from various locations across the United States and clinical histories (2). These isolates

were sequenced and shown to be closely related to SVV-001, supporting the role of swine as a natural host. Knowles et al. used two of the historical isolates to inoculate pigs, but was unsuccessful in demonstrating specific clinical disease (2).

In 2007, a load of market hogs being transported from Canada into the United States contained animals presenting with lameness and vesicular lesions on the snouts and coronary bands (6). These animals were diagnosed with porcine idiopathic vesicular disease (PIVD) after testing negative for the top differentials of infectious causes of vesicular disease in swine including foot-and mouth disease virus (FMDV). Interestingly, samples from these animals did test positive for SVA by reverse transcriptase polymerase chain reaction (RT-PCR). In 2012, Yang et al. inoculated four pigs with a SVA isolate received from NVSL. Although all pigs developed a viremia and an antibody response, no specific clinical disease was observed. Subsequently, a case report of a single boar in Indiana with vesicular disease was published and again samples tested PCR positive for SVA (7). Thus, experimental reproduction of specific disease with SVA continued to be unsuccessful, but field cases provided additional evidence vesicular disease was associated with SVA infection in pigs.

Starting in late 2014 and into 2015 several states in Brazil reported a sharp increase in cases of PIVD and an increase in pre-weaning mortality (8, 9). Samples from these cases tested positive for SVA, which was the first detection of the virus in Brazil. Subsequently, similar cases emerged in the United States in the summer of 2015 (10, 11). With mounting evidence supporting SVA as a causative agent for vesicular disease in swine, multiple experimental studies were performed and reproduced vesicular disease after SVA inoculation (12-14). The success in demonstrating disease across various age groups, led to questions regarding previous attempts that were unable to show specific disease in pigs exposed to SVA. Some areas of

speculation were differences in pathogenicity of the viruses, susceptibility of the host, or the requirement of a novel co-factor.

Since questions still remain about the pathogenicity of historical isolates, the objectives of this study were to detail infection dynamics of three historical and three contemporary SVA isolates in swine, characterize cross-neutralizing antibody responses, and compare SVA genomic sequences. The hypothesis was that historical SVA strains isolated prior to the 2015 outbreak in the US would be less pathogenic than contemporary isolates in swine.

Materials and Methods

Cells and virus

A swine testicular (ST) cell line was cultured at 37 °C and 5% CO2 in Minimum Essential Medium (MEM, MilliporeSigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, AtlantaBio, Flowery Way, GA), gentamicin (VetOne/MWI, Boise, ID) and L-glutamine (Life Technologies, Carlsbad, CA). ST cells were used for virus titration and virus neutralization assays. Although ST cells may not grow SVA to as high of titers as other susceptible cell lines (15), they were utilized due to their susceptibility and the ease of reading cytopathic effect (CPE) microscopically (16).

Six isolates of SVA were used in the study. A brief history of each isolate is as follows. SVV001/2002 was the original cell culture contaminant strain SVV-001 purchased from the American Type Culture Collection (ATCC PTA-5343) and was passaged in cell culture three times (p3). CAN/2011 was obtained from NVSL originally isolated from a swine brain with origins in Canada and a history of vesicular disease (p2). HI/2012 (NADC40) was isolated from serum of a pig in Hawaii exhibiting vesicular disease by the Agriculture Research Service National Animal Disease Center (NADC) (p2). SD/2015 was isolated from vesicular fluid of a pig inoculated with SVA15-41901SD (p2) (10, 17). IA/2015 was isolated from samples collected

from a vesicular disease case in finishing pigs located in Iowa (p7). NC/2015 was isolated from a swine sample submitted to the Kansas State Veterinary Diagnostic Laboratory from North Carolina with no clinical history of vesicular disease (p4). Subsequent next-generation sequencing (NGS) and PCR revealed the presence of porcine respiratory coronavirus contamination in the viral stock of NC/2015. NC/2015 was received with a titer around 105 TCID50/mL, while the remaining isolates had titers around 10^7 $TCID_{50}$/mL calculated using the Reed and Muench method (18). Therefore, the viral stock of NC/2015 was diluted 1:2 with MEM, while the remaining viruses were diluted 1:5 with MEM for inoculum preparation (5 mL/animal).

Animals and experimental design

Study pigs were farrowed at the NADC from sows purchased from a commercial source in Wisconsin, USA. All research was performed in accordance with an approved NADC Animal Care and Use Protocol (ACUP 2867). All pigs were clinically free of lesions, negative for SVA by PCR in serum and rectal swabs, and seronegative at the start of the study. Animals were housed under ABSL-2 conditions for the experiment. Fifty-four pigs ranging in age from 5-6 months were placed into one of six treatment groups (n=8 per group) and one control group (n=6) based on ear tag number blocking by litter. Each treatment group was housed in a separate room for the experiment.

Animals in treatment groups were inoculated intranasally with 5 mL of inoculum around 10^7 $TCID_{50}$/mL except for the NC/2015 group that received 10^5 $TCID_{50}$/mL on 0 days post inoculation (0 dpi) split between both nostrils. Animals were bled and oral swabbed on 0, 4, 7, and 14 dpi. Rectal swabs and observations for vesicle development and lameness were performed daily between 0-11 and on 14 dpi. All animals were humanely euthanized at 14 dpi with an intravenous dose of barbiturate (Fatal-Plus® Solution, Vortech Pharmaceuticals,

Dearborn, MI) according to the manufacturer label dose. One animal in the SVV001/2002 group had to be euthanized prior to the start of the study due to health issues.

Sample collection and lesion scoring

Pigs were manually restrained for blood collection via jugular venipuncture and blood was transferred into serum separator tubes (BD Vacutainer®, Franklin Lakes, NJ). Tubes were centrifuged for 10 min at 920 x g to harvest the serum. Oral and fecal swabs were collected using a sterile polyester tipped applicator (Puritan Medical Products, Guilford, ME), immersed in 3 mL of serum-free MEM. All samples were stored at -80 °C for future testing.

Vesicular lesions present on the coronary bands and snout were scored with a 5 point system: each foot that displayed a vesicular lesion was given 1 point and a vesicle on the snout was assigned 1 point. An animal was given a score of zero if no lesions were identified. Average lesion score was calculated by adding up the scores for each pig in a group divided by the total number of pigs in the group.

SVA nucleic acid quantification

Serum and swab samples were extracted and tested by real-time RT-PCR (RT-qPCR) as previously described (17). Briefly, RNA was extracted from samples using the MagMAX™ Pathogen RNA/DNA kit (catalog no. 4462359; Applied Biosystems, Waltham, MA) following manufacturer's recommendations. Next, 5 µL of extracted product was added to 20 µL of the Path-ID™ Multiplex One-Step RT-PCR reaction master mix for fecal swabs or 20 µL AgPath-ID™ One step RT-PCR kit for oral swabs, vesicle swabs, and sera (Applied Biosystems). The primers and probe were designed to target a conserved region containing nucleotides 602-710 of the SVA genome. The forward primer sequence was 5'-TGCCTTGGATACTGCCTGATAG-3', the reverse primer sequence was 5'-GGTGCCAGAGGCTGTATCG-3' and the probe sequence

was 5'-CGACGGCCTAGTCGGTCGGTT-3'. RNA copies were calculated based on a plasmid containing the target region and Ct values greater than 35 were considered negative.

Virus neutralization (VN) assay

Sera from challenged animals were tested by VN assay to determine neutralizing antibody response to the challenge strain in addition to cross-neutralizing titers against all other viruses utilized in the study. Serum samples were heat-inactivated at 56 °C for 30 min, serially diluted 1:4 (up to 1:4096) in MEM, and run in quadruplicate. An equal volume of diluted SVV001/2002, CAN/2011, HI/2012, SD/2015, IA/2015, or NC/2015 (~200 $TCID_{50}$) was added to the serum and incubated for 1 hour at 37 °C. The virus-serum mixture was transferred to 96-well plates of ST cells grown to a confluent monolayer containing MEM supplemented with 2% FBS. Plates were microscopically evaluated for cytopathic effect (CPE) daily for 4 days. Titers were recorded as the highest dilution of serum at which the infectivity of the SVA isolate was completely neutralized in at least 50% of the inoculated wells. VN assays using each SVA isolate were performed on all 14 dpi serum samples during one run with a back-titration of the viral solution. Viruses were titrated during each run and ranged from 30-500 $TCID_{50}$/well. Sera from 0 dpi was only tested against the homologous strain of SVA. Virus neutralization titers of ≤1:16 were considered negative (19). The geometric mean of the 7 or 8 pigs in each treatment group was calculated for comparison of cross-neutralizing titers.

Sequencing

One sample from each of the six treatment groups was selected for virus isolation based on the highest number of genomic copies present in either serum or swabs as determined by RT-qPCR (4-7dpi). To ensure adequate viral genetic material was present for sequencing, supernatant from cell cultures was collected 48 hours after a monolayer of ST cells was inoculated with sample and total RNA was extracted from the supernatant of the second passage

using the NucleoSpin Virus Kit (Clontech, Takara Bio Inc.). A RT-PCR assay was performed using primers designed from the original SVV-001 sequence in GenBank (NC_011349). PCR products were run on an agarose gel to purify bands of interest. Bands were removed and fragments were cloned into a pCR™4Blunt-TOPO® vector (catalog no. 45-0031, Invitrogen). Cloned products were then sent to Iowa State University DNA Facility and the sequencing facility at NADC for Sanger sequencing. Sanger runs were done in duplicate from each direction using multiple plasmids for each sequenced segment. Any gaps remaining in the sequence were filled by developing new specific primers for the target and repeating the same procedure to complete the sequence (Supplemental Table 1). The 3' and 5' ends of the sequences were completed using 3' (TAKARA SMARTer® RACE 5'/3' Kit) and 5' (Invitrogen™ 5' RACE System for Rapid Amplification of cDNA Ends, version 2.0) RACE kits according to the manufacturer instructions. Sequences have been added to GenBank (MT360257-MT360262).

Sequences of challenge isolates (i.e., inocula) were also obtained for comparison to Sanger sequencing of animal isolates. Three inoculum strains had already been deposited into GenBank: SVV001/2002 (KU954086), CAN/2011 (KC667560), and SD/2015 (KU051394). The sequence for IA/2015 was provide by the collaborator. Cell culture supernatant samples from HI/2012 and NC/2015 were sent to Iowa State University for next generation sequencing (NGS) using the Illumina platform. In addition, the animal viral isolate for CAN/2011 was sent for NGS sequencing for confirmation due to laboratory error during the Sanger sequencing process. Library preparation, sequencing, and sequencing data analysis methods were performed following previously published methods (20, 21).

Analysis

Data analyses and graphic representations were performed by using Microsoft Office Excel 2010 (Microsoft, Bellevue, WA, USA) and GraphPad Prism 8.1.2 (San Diego, CA, USA),

respectively. Complete genome nucleotide sequences were first generated from sequence reads using Lasergene 11 (DNASTAR, Inc., Madison, WI, USA). Illumina NGS sequence reads were assembled and analyzed, and final nucleotide alignments were generated using the MAFFT algorithm (22, 23), in Geneious Prime Version 2020.0.4 (Biomatters Ltd, Auckland, New Zealand). Phylogenetic examination was completed using RAxML using the GTR Gamma Nucleotide Model and the Rapid Bootstrapping (x500) and search for the best-scoring Maximum Likelihood Tree Algorithm with a random parsimony seed of one (24) available in Geneious. The output tree was imported into FigTree v1.4.3 for optimal display (http://tree.bio.ed.ac.uk/software/figtree/).

Venkataraman et al. reported the residues from each of the structural proteins that make up the most prominent loop structures of SVV, which can help determine cell tropism for picornaviruses as well as antigenic sites that elicit immune responses to infection (3, 25). The prominent loops analyzed for AA changes between the six SVA isolates in this study are as follows: BC loop of VP1 (VP1 residues 50-74, P1 residues 724-748), Loop II or CD loop of VP1 (94-109, 768-783), GH loop of VP1 (185-215, 859-889), “the puff” of VP2 (172-200, 323-351), and “the knob” of VP3 (57-73, 492, 508).

Results

Clinical disease

Animals from every treatment group developed clinical signs of vesicular disease, while control animals did not. No other clinical signs such as lameness, lethargy, or inappetence were observed during the study. Vesicular lesions on the coronary band were more common than those on the snout. Only 2 pigs in this study developed snout lesions. The number of animals that developed lesions in each group ranged from 5-8 pigs (62.5%-100%). Vesicular lesions were

visually similar between pigs infected with historical isolates compared to contemporary isolates (Figure 1A-C).

A score out of five was assigned to each animal to score gross lesions and an average was calculated for all pigs in each group daily (Figure 1D). All pigs (8/8) inoculated with SD/2015 and HI/2012 developed lesions and these isolates had the highest average lesion score by the end of the study. The lowest average lesion score was in pigs inoculated with NC/2015 with 6/8 pigs developing vesicles. There was a range in onset of vesicle development between the 6 treatment groups. The two oldest isolates in the study, SVV001/2002 and CAN/2011, had the fastest onset of clinical signs with the first pig to develop a vesicle at 3 and 2 dpi, respectively. The two viruses with the slowest onset of lesion development were SD/2015 and HI/2012 starting at 7 and 9 dpi respectively. Pigs inoculated with IA/2015 and NC/2015 developed clinical signs beginning at 4 dpi.

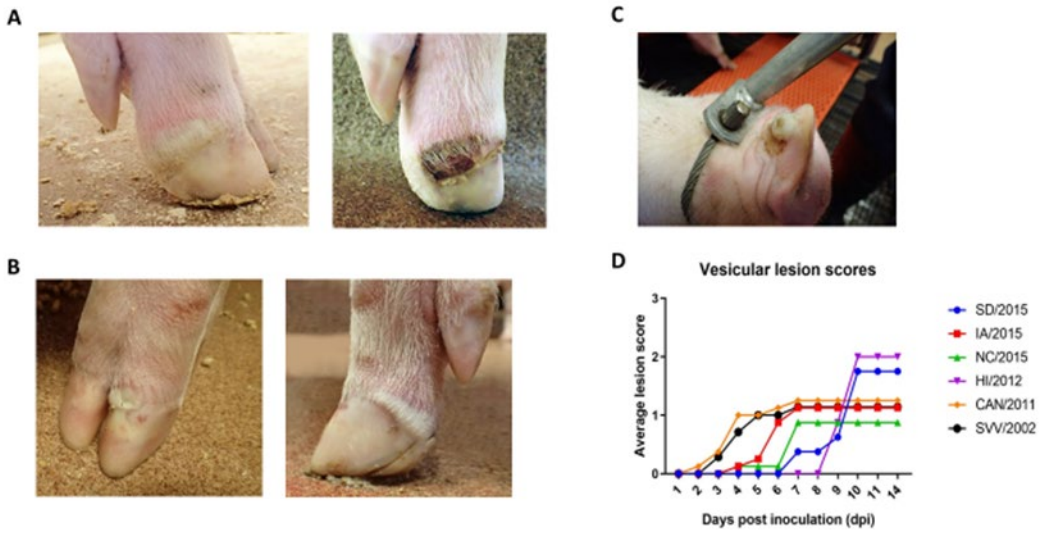

Figure 4.1: Clinical disease in inoculated animals. A) Intact coronary band lesion observed in a pig infected with a historical isolate on 2 dpi and ruptured coronary band lesion in another pig on 5 dpi. B) Intact coronary band lesion observed in a pig infected with a contemporary isolate on 5 dpi and an intact coronary band lesion in another pig on 7 dpi. C) Snout lesion on a pig infected with a contemporary isolate on 5 dpi D) Animals were scored on a scale of 0-5. Average lesions scores plotted for each group.

SVA RNA Detection

RT-qPCR was performed on serum and swab samples. All selected samples from control animals tested negative for SVA RNA by PCR. All animals in treatment groups were negative for SVA in all samples on 0 dpi.

Peak viremia for all groups occurred at the 4 dpi sample except NC/2015, which peaked at the 7 dpi sample time point (Figure 2A). None of the animals in the study remained viremic on 14 dpi. All animals in SD/2015, NC/2015, and HI/2012 groups had a detectable viremia on at least one sampling time point. In the CAN/2011 group 6/8 animals had PCR positive serum. Finally, only 4/8 and 3/7 animals in IA/2015 and SVV001/2002 treatment groups, respectively, had a detectable viremia during the study. The lowest levels of viremia were detected in pigs challenged with SVV001/2002.

RNA levels in oral swabs peaked during the first week after inoculation with levels reducing on 14 dpi (Figure 2B). All animals in treatment groups had at least one RT-qPCR-positive oral swab and a majority of the animals still had positive oral swabs on 14 dpi. In addition, all animals in this study had a least one PCR-positive rectal swab. CAN/2011 animals had high levels during the first few days after inoculation and peaked at 5 dpi (Figure 2C). SVV001/2002 had a similar levels compared to CAN/2011. In contrast, SD/2015, NC/2015, and HI/2012 peaked later in the study around 10 dpi and had the higher levels in the second half of the study (Figure 2D).

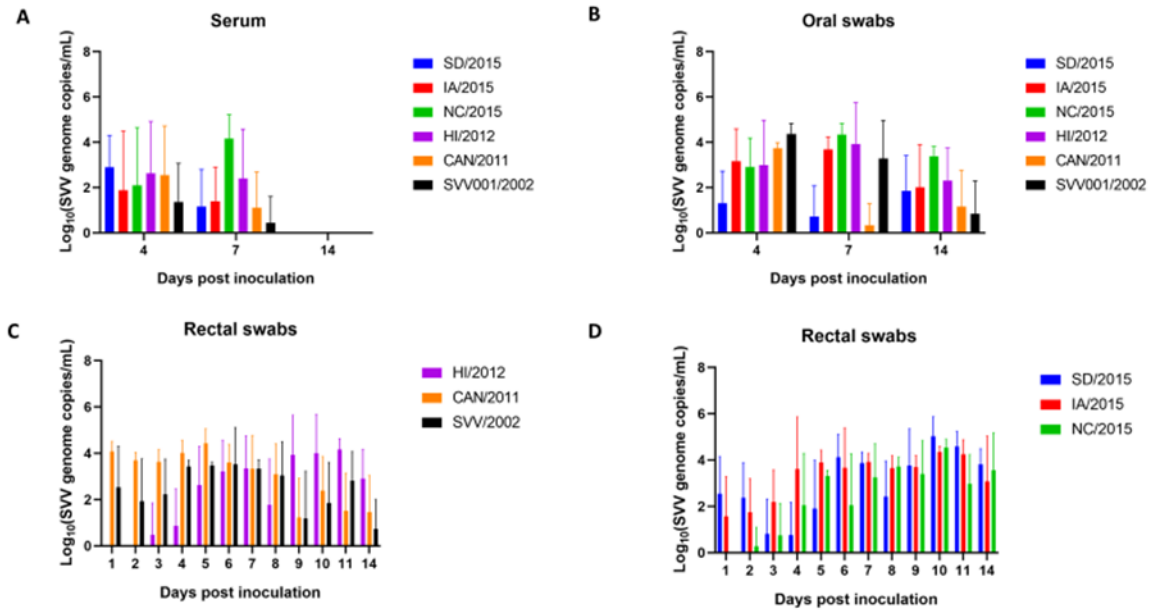

Figure 4.2: Viral load as detected by RT-qPCR in A) serum, B) oral swabs, and C,D) rectal swabs. Rectal swab results were split into a graph for historical isolates (C) and a graph for contemporary isolates (D). Bars represent a mean of all 7 or 8 pigs in a group. Error bars are standard error of the mean. The legend contains the color code to all six viruses.

SVA neutralizing antibodies

A virus neutralization assay was run on 0 and 14 dpi serum for all animals to assess neutralizing and cross-neutralizing titers. All animals had titers ≤1:16 (i.e., seronegative) on 0 dpi and developed a neutralizing antibody response against the homologous isolate in addition to cross-neutralizing titers against all other isolates used in this study by 14 dpi (Table 1). A geometric mean was calculated for all animals in each group. Pigs inoculated with SVV001/2002 had the lowest cross-neutralizing titers compared to animals inoculated with other strains, though serum from the CAN/2011 group also had low cross-neutralizing titers. Serum from pigs inoculated with SD/2015 and IA/2015 performed similarly on the VN assay against all viruses. Serum from pigs inoculated with NC/2015 and HI/2012 also performed similarly against all

viruses. Most treatment groups had high neutralizing titers against SD/2015 and IA/2015. In contrast, most groups had low cross-neutralizing titers to SVV001/2002, HI/2012, and NC/2015.

Table 4.1: Virus neutralization assay results for 14 dpi serum against all six viral isolates.

Pig antiserum	SD/2015*	IA/2015	NC/2015	HI/2012	CAN/2011	SVV/2002
SD/2015#	1722^	2896	1448	1448	609	464
IA/2015	2048	2048	1024	1722	724	380
NC/2015	431	512	609	724	152	64
HI/2012	609	609	431	724	181	95
CAN/2011	609	861	1024	1448	1024	210
SVV/2002	215	304	431	362	304	1024

*Columns represent virus used to inoculate pigs
#Rows represent virus used in the virus neutralization assay
^Numbers in the boxes represent the geometric mean of the titer from virus neutralization assay results for all pigs in each group.

SVA sequence comparison and phylogenetics

The complete genome was sequenced from virus isolated from animal samples representing all 6 treatment groups in this study via Sanger sequencing methods to demonstrate the virus being shed from the animals was the same virus used for inoculation. The sequences obtained from animal samples had 99.8% nucleotide identity or greater to their respective inoculum strains sequenced by next-generation sequencing (NGS). Three inoculum strains had already been deposited into GenBank: SVV001/2002 (KU954086), CAN/2011 (KC667560), and SD/2015 (KU051394). The percent nucleotide identity among all isolates was 93.7% or greater (Table 2). If SVV001/2002 and CAN/2011 (95.1% nucleotide identity) were removed the remaining isolates would have a 97.9% nucleotide identity. SD/2015 and IA/2015 were the most similar with a nucleotide identity of 99.2% with a difference of only 58 nucleotides.

SVV001/2002 and IA/2015 had the most differences with a nucleotide identity of 93.7% and a difference of 456 nucleotides with those differences spread throughout the genome. A comparison of the six SVA isolates to a consensus sequence can be seen in Figure 3, which also supports the conclusion that SVV001/2002 and Can/2011 share a low nucleotide identity with the remaining four isolates.

Table 4.2: Distance matrix and heat map for the SVA genomes included in this study.

	SVV/2002	Can/2011	HI/2012	NC/2015	SD/2015	IA/2015
SVV/2002		357*	403	430	447	457
Can/2011	95.1#		219	270	283	293
HI/2012	94.5	97.0		145	149	154
NC/2015	94.1	96.3	98.0		89	101
SD/2015	93.9	96.1	98.0	98.8		58
IA/2015	93.7	96.0	97.9	98.6	99.2	

* Numbers above the 100% nucleotide identity bars (black) are the numbers of nucleotide differences between the genomes
#Numbers below the black bars are % nucleotide identities between the genomes

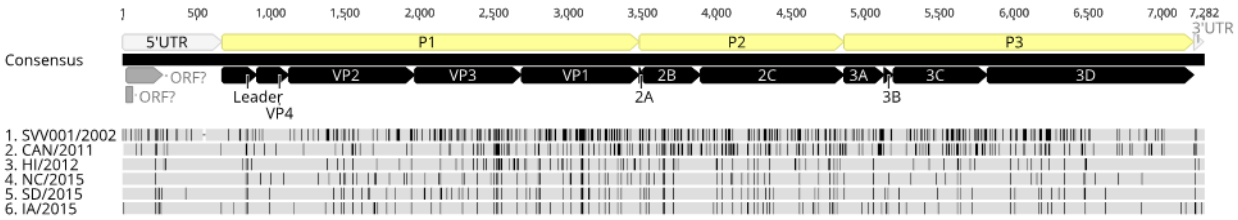

Figure 4.3: Graphic representation of the SVA genome comparisons to the consensus sequence. Nucleotides representing the 5'-UTR and primary polyproteins (P1, P2, P3) are indicated above, and the predicted final processed ORFs are shown below the consensus sequence. Vertical lines represent differences when compared to the consensus sequence when viewed with a strict threshold of 50%. One undescribed open reading frame (ORF?) defines an ORF of 249 nt (83 aa) in sequences #2-6, and a much smaller ORF of 36 nt (13 aa) in sequence #1 (SVV001).

Prominent loop structures in the capsid of SVA can help determine receptor binding and serve as B cell epitopes. AA changes between the six isolates in these structures are as follows. The BC loop of VP1 (residues 724-748) had 3 non-conservative AA changes between the isolates compared to the consensus sequence (Figure 4). Two changes were present in SVV001/2002, while CAN/2011, HI/2011, and IA/2015 had one change. Only one non-conserved AA difference was found in Loop II (CD loop, residues 768-783) of VP1 again in SVV001/2002. The GH loop of VP1 (residues 859-889) and "the puff" of VP2 (residues 323-351) did not have any non-conservative AA differences between the isolates. Finally, "the knob" of VP3 (residues 492-508) had 4 non-conservative AA differences from the consensus sequence. All four positions could be found in SVV001/2002. CAN/2011 and HI/2012 had 1 AA change the same as SVV001, but in contrast the contemporary isolates all had a different AA change in the same position.

Phylogenic analysis was completed after all available full-length genomes (220) in GenBank were downloaded and aligned (03/30/2020). The midpoint rooted dendrogram revealed a major shift in phylogeny between 2002 and 2005 and development of a new clade of viruses (Figure 5). Clade 1 represents older SVA isolates and Clade 2 represents newer viruses. SVV001/2002 is located in Clade 1 while the remaining 5 isolates are found in Clade 2. The other five isolates are spread throughout Clade 2, though IA/2015 and SD/2015 fall closer together than the others. Interestingly, NC/2015 fell into the same branch as an isolate from Colombia from 2016. SVV001/2002, CAN/2011, and SD/2015 were grouped with the viruses that were used to challenge those animals and had already been sequenced and deposited into GenBank.

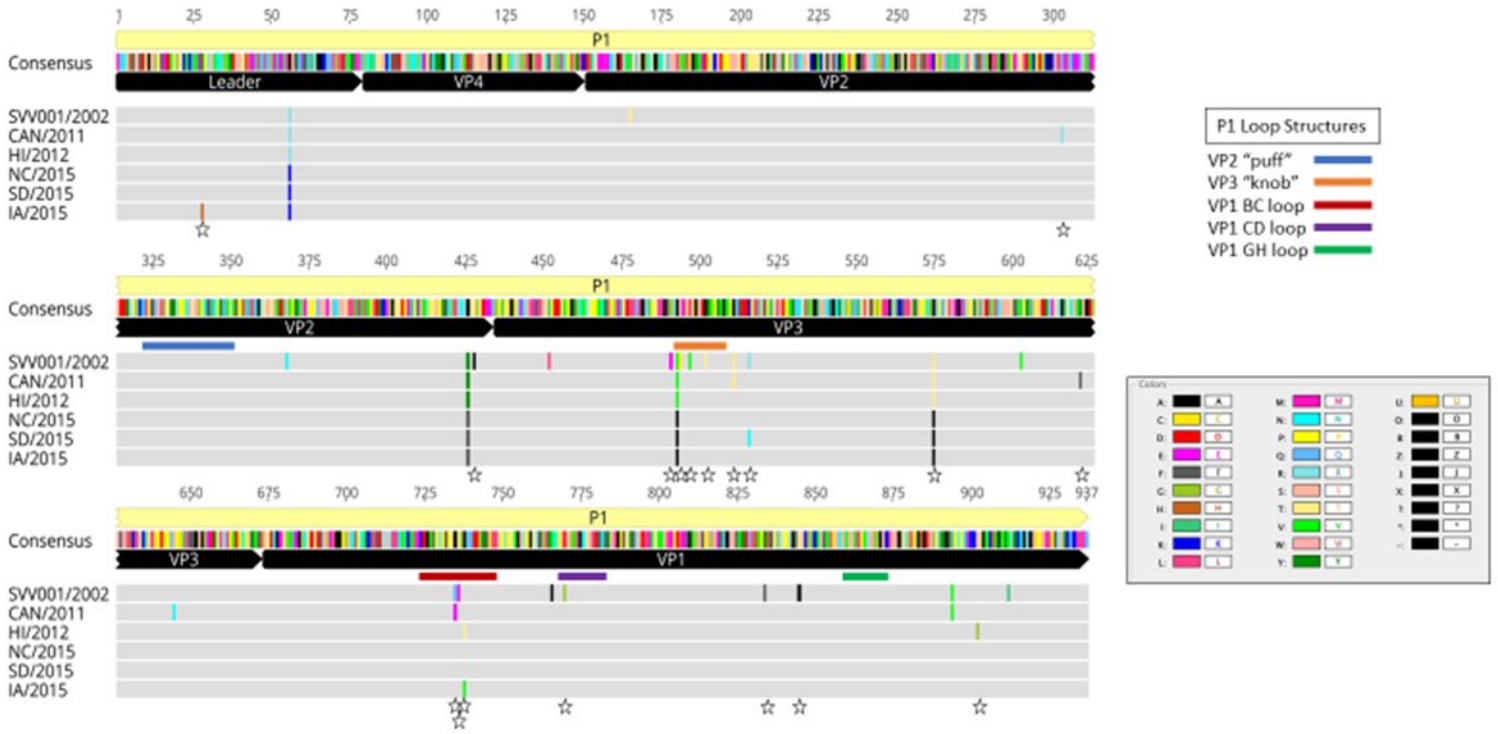

Figure 4.4: Graphic representation of the amino acid alignment for SVA P1 protein region. There are many other amino acid differences elsewhere in the genome. The consensus sequence is color coded by amino acid residue and colors are shown in the legend. Prominent loop structures are also indicated. The amino acids representing primary polyproteins P1 is indicated above, and the predicted final processed VPs are shown below the consensus sequence. Vertical lines represent differences when compared to the Consensus sequence when viewed with a strict threshold of 50%. Non-conservative amino acid changes are indicated by a star (☆).

Figure 4.5: Phylogenetic analysis (midpoint rooted, increasing node order) of 220 SVA genomes available in GenBank along with the six genomes sequenced in this study. CA2011, HI2012, NC2015, SD2015, IA2015 are shown in blue typeface, while the re-sequenced index strain SVV-001/2002 is shown in green typeface. Clade 1 includes all older isolates, while Clade 2 represent newer isolates.

Discussion

Recently, Fernandes et al. compared pigs inoculated with the original cell culture contaminant, SVV-001, to those inoculated with a US contemporary isolate from 2015 (26). They reported that pigs inoculated with the historical isolate did not show signs of vesicular disease, but inoculation did result in infection and antibody response (26). In addition, another study compared the pathogenicity of two Chinese contemporary isolates (2016 vs 2017) and found one isolate to be more pathogenic in pigs than the other (27). To further explore pathogenicity differences between SVA strains, this study compared three strains isolated prior to the outbreak of SVA in both Brazil and the US in 2015 and three more isolated during the outbreak.

Whether pigs were inoculated with a historical isolate or a contemporary isolate, a majority of the animals in each treatment group developed vesicular lesions. In the SVV001/2002 group, 5/7 animals developed clinical signs of vesicular disease. This study is in contrast to a recent pig study that reported they did not observe vesicular disease in those animals inoculated with a SVV-001 isolate, though they did observe lesions in animals inoculated with a 2015 SVA isolate (26). Both research groups purchased the SVV-001 isolate from ATCC. In this study 5-6-month-old pigs were challenged intranasally with 5 mL of inoculum with a titer of $>10^5$ $TCID_{50}$/mL, while the other study challenged 4-month-old pigs oronasally with 10 mL of inoculum (5 mL orally and 5 mL intranasally) with a titer of $10^{7.5}$ $TCID_{50}$/mL. Both studies had experimental groups of similar size and animals close in age, though genetic differences are unknown. Fernandes et al., used H1299 cells, a human cancer cell line, to grow the virus while this study used a swine cell line (ST cells). Passage on swine cells versus human cells could have aided virus adaptation to pigs. In addition, ST cell culture supernatant collected for viral stock solutions may contain a factor that contributes to virus pathogenicity. These differences may

have contributed the contrasting reports of clinical disease development after challenge with SVV-001.

Field observations of SVA infections at farms have reported a large range of percentage of animals developing vesicular lesions during an outbreak (8, 28). It has also been reported that clinically affected sows had a similar proportion of PCR positive samples compared to non-clinically affected sows (29). Thus, providing evidence that even current circulating isolates of SVA in the field do not cause clinical disease in all infected animals, which is supported by a seroprevalence study in the US suggesting SVA is circulating subclinically (30). In addition to viral factors, there could be external factors such as skin abrasions or friction that contribute to the development of vesicular lesions and location whether on the coronary bands or snout. Pigs in this study were housed on a flat concrete floor which may have been more abrasive than other flooring used in animal research housing such as raised decks. NC/2015 was isolated from a diagnostic sample from pigs not showing clinical signs of vesicular disease, but in this study when inoculated into pigs it did cause vesicular disease. Further work should be performed to understand viral pathogenesis and why some animals develop lesions after SVA infection, while others do not.

Since the inoculums utilized in this study did not have identical titers, it is difficult to make direct comparisons of quantitative PCR results from serum and swabs, but some general conclusions can be drawn. All animals in this study replicated virus and had detectable nucleic acid in serum, oral swabs and/or rectal swabs. This is in agreement with previously reported work with SVV-001 and a 2015 isolate; though, they also reported lower virus levels in serum in pigs challenged with SVV-001, but similar levels in oral and fecal swabs (26). This study similarly found lower levels of virus in the blood of pigs inoculated with SVV001/2002

compared to other treatment groups in the study. Since, this virus was discovered as a cell culture contaminant it may be cell culture adapted and replicate less efficiently in pigs compared to the other isolates that were obtained from pig samples. This research supports previous work reporting a short viremia and in this study some pigs did not develop a detectable viremia after inoculated with SVA, which indicates that serum may not always be relied upon alone to detect SVA infection (13, 31).

Interestingly, CAN/2011 had the highest levels of virus detected by PCR in rectal swabs for the first 3 days after inoculation and this group was the first to develop clinical signs of vesicular disease. In addition, pigs in both HI/2012 and SD/2015 groups were the latest to develop vesicles and timing coincided with later peaks in shedding of virus detected by rectal swabs. High levels of virus detected in rectal swabs may coincide with higher levels of viral replication in keratinocytes of the epidermis of the coronary bands and snout leading to vesicle development. Due to the timing of increased shedding in rectal swabs with lesion development in some groups, rectal swabs may be a reliable sample to collect besides lesion swabs during a disease break when lesions are observed.

VN results demonstrated that animals developed neutralizing antibody titers against the homologous virus as well as cross neutralizing titers against heterologous viruses used in the study suggesting SVA has a single serotype. SVV001/2002 animals had the lowest cross-neutralizing antibody titers. Fernandes et al., also reported lower neutralizing antibody titers in pigs inoculated with SVV-001 compared to a 2015 isolate (26). SVV001/2002 is the most genetically distinct from the other viruses used in this study, thus amino acid changes in the capsid proteins resulting in altered neutralizing epitopes could explain the lower cross-neutralizing titers. It is still unknown what titer of neutralizing antibody provides protection for

an animal, so lower titers could still provide protection against viral challenge. In addition, others have studied the cell-mediated immune response, which also plays a role in protection (26, 31). Future studies utilizing heterologous challenge models would help discern if lower cross-neutralizing antibodies are still protective against challenge.

Sequencing results supported that the inoculum virus was the same virus that was isolated from animal samples. Sequences from animal isolates only had a few base substitutions compared to the inoculum, thus showing that one passage in a pig and a passage through cell culture did not drastically alter the viral genome. It has been reported that RNA viruses have a mutation rate ranging from 10^{-6} to10^{-4} substitutions per nucleotide per cell infection (s/n/c) (32). Information from these 2 passages support that rate. SVV001/2002 is the only isolate that was not collected directly from a swine sample and was older than all other isolates by ten years. The combination of these factors and others may explain its genetic distance from other SVA isolates.

Although these 6 viruses are genetically similar, the AA sequences of some of the most prominent loop structures of the structural proteins on the capsid of the virus did have some AA differences. Though there were not many AA changes, they could play a role in altering structure and explain some of the differences observed in neutralizing titers reported in this study. The BC loop and loop II of VP1, “the puff” of VP2, and “the knob” of VP3 were picked out for further evaluation since they have been shown to interact with the SVA receptor ANTXR1 and similar regions have been shown to be involved in neutralizing epitopes for FMDV and other picornaviruses (33, 34). Other linear B cell epitopes on VP1 and VP2 have recently been reported; thus, further examination of changes between SVA isolates in the capsid proteins is necessary (35).

Although previous studies were unable to experimentally reproduce vesicular disease in swine after SVA inoculation with historical isolates, pigs inoculated with SVV001/2002, a cell culture isolate, developed vesicular disease in this study. This research also demonstrated two other isolates from prior to 2015 were able to induce vesicular lesions similar to contemporary isolates; however, these strains were isolated from pigs that presented with lesions. Conflicting reports about the ability of SVV-001 to cause clinical disease support the need for additional research to better understand viral factors contributing to pathogenicity of SVA and host factors contributing to development of vesicular disease in swine.

Acknowledgments

The authors wish to thank Dr. Ganwu Li and his staff at Iowa State University's Veterinary Diagnostic Laboratory for next-generation sequencing support. In addition, Deb Adolphson and Sarah Anderson for their technical assistance and Jason Huegel, Justin Miller, and Keiko Sampson for their animal care and handling. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

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CHAPTER 5. INFECTIOUS DOSE OF SENECAVIRUS A IN FINISHING PIGS AND NEONATES

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Modified from a manuscript to be submitted to *PLoS One*

Abstract

Senecavirus A (SVA) is a picornavirus that causes vesicular disease in swine. With the spread of SVA around the globe, some countries free of foot-and-mouth disease (FMD) are experiencing an endemic vesicular disease in their swine populations, which adds to the time and cost burden of ruling out FMD every time a vesicle is observed since FMD and SVA are clinically indistinguishable. Understanding the pathogenesis of the virus and its ability to transmit to naïve populations is critical to formulating control and prevention measures. The primary objective of this study was to determine the infectious dose of SVA in finishing pigs and neonates. A 2011 SVA isolate was serially hundred-fold diluted to create four challenge inoculums ranging from $10^{6.5}$ to $10^{0.5}$ $TCID_{50}$/mL. Four finishing pigs individually housed were intranasally inoculated with 5 mL of each dose (n=16). For neonates, serial ten-fold dilutions were used to create 6 challenge inoculums ranging from $10^{5.5}$ to $10^{0.5}$ $TCID_{50}$/mL. Again, four animals in individual housing were challenged orally with 2 mL of each dose (n=24). Detection of SVA by PCR in collected samples and/or neutralizing antibody response was utilized to classify an animal as infected. The minimum infectious dose (MID) for this study in market

weight animals was 1,260 $TCID_{50}$/mL ($10^{3.1}$ $TCID_{50}$/mL) and similarly for neonates it was 316 $TCID_{50}$/mL ($10^{2.5}$ $TCID_{50}$/mL). Information on the infectious dose provides information that can guide biosecurity and disinfections measures to control the spread of SVA.

Introduction

Senecavirus A (SVA) is a small, non-enveloped, single-stranded, positive-sense RNA virus in genus *Senecavirus* and family *Picornaviridae* (1). It was originally discovered as a cell culture contaminant in 2002 and named Seneca Valley virus (SVV-001); although, similar picorna-like viruses had been identified in swine samples dating back to the late 1980s suggesting circulation of the virus in swine (2, 3). Early experimental inoculation of pigs resulted in viral replication and an antibody response but did not produce any specific clinical disease (2, 4). In late 2014, vesicular disease outbreaks including increased neonatal mortality on sow farms occurred in Brazil and similar cases were reported in the United States (US) in 2015 with diagnostic testing confirming the presence of SVA (5-8). Evidence from the field and experimental reproduction of vesicular lesions in pigs from weaning to breeding age demonstrated that SVA was a causative agent for vesicular disease in swine (9-12).

In addition to vesicular disease, both Brazil and the US reported increases in neonatal mortality on affected farms with clinical signs including lethargy, wasting, and occasionally diarrhea, but Brazil also reported vesicular lesions and neurologic signs in neonates not commonly observed in the US (13-15). Further testing in many cases ruled out other causative agents for the clinical signs observed, thus leading to the conclusion that SVA was also the cause of mortality in piglets on affected breeding farms. Currently, there have been no published reports of experimental reproduction of neonatal morbidity or mortality with SVA.

SVA has been isolated from environmental samples on swine farms including dust from fans, hallways in barns, and even the ground outside barns providing evidence of humans serving as fomites to spread the virus around swine facilities (16). In addition, it has been isolated from environmental samples taken from an assembly yard for animals prior to shipment to slaughter (17). A recent study found a significant number of cull sows entered multiple collection points prior to reaching a slaughter facility making the cull sow network a likely area of pathogen transmission (18). Not only has SVA been found on environmental surfaces, but also in mouse feces and flies providing additional vectors for the spread of virus (16). Demonstration of virus in the environment by both PCR and virus isolation has led to the question of whether this virus could be infectious to pigs and play a role in transmission.

Understanding the epidemiology of SVA and ways to control or prevent spread is important considering it causes clinical disease identical to foot-and-mouth disease virus (FMDV). FMDV is an economically devastating disease not only for FMDV-free countries with an introduction event, but also for those countries with endemic virus battling production losses and vaccination costs (19). Since FMDV is on the World Organization for Animal Health (OIE) list of notifiable diseases, having an endemic vesicular disease in a country free of FMDV also costs time and money since every time a vesicular lesion is observed it must be investigated to rule out FMDV (20). In the US, cases of vesicular disease due to SVA have been an issue with secondary and cull sow markets where collection points are continuous flow and animals are consistently co-mingled allowing virus spread and persistence (18).

Current environmental testing by PCR for SVA can be difficult to interpret due to the lack of information about the infectious dose of SVA. The main objective of this study was to determine the minimum infectious dose of SVA in both finishing pigs and neonates. An

additional goal was determining the correlation between PCR Ct values, tissue culture infectious dose (TCID), and swine bioassay. Results from this study can provide context for interpreting SVA PCR results especially those collected from environmental samples.

Materials and Methods

Cells and Virus

Swine testicular (ST) cells were grown in minimum essential medium (MEM, MilliporeSigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, AtlantaBio, Flowery Way, GA), 1% L-glutamine (Life Technologies, Carlsbad, CA), and gentamicin at 37 °C and 5% CO2. ST cells were used for virus growth, titration, and virus neutralization assays. The virus used for animal inoculation was supplied by the National Veterinary Services Laboratory (strain 11-55910-3). It was isolated from a swine brain sample collected from a group of market hogs traveling to slaughter from Canada to the US that presented with lameness and vesicular disease (GenBank KC667560). The isolate was propagated on ST cells with the second and third passage being pooled and diluted to a titer of $1x10^{6.5}$ $TCID_{50}$/mL, which was termed the stock virus (SVA/CAN/2011).

Inoculum and virus titration

Inoculum for the finishing pigs was made by serial hundred-fold dilutions of the stock virus with MEM to create four challenge inoculums ranging from $10^{6.5}$ to $10^{0.5}$ $TCID_{50}$/mL. Inoculum for the neonates was made by serial ten-fold dilutions of the stock virus with MEM to create 6 challenge inoculums ranging from $10^{5.5}$ to $10^{0.5}$ $TCID_{50}$/mL.

Virus titrations were performed on confluent ST cells in a 96-well plate with each well containing 100 µL of growth medium. Virus stocks were serially ten-fold diluted in MEM and 100 µL was added to seven wells in the column of a plate with the remaining well serving as a

control for each virus dilution. Plates were incubated and examined microscopically for cytopathic effect for four days after inoculation. Viral titers were calculated using the Reed and Meunch method (21).

Animal study design

All animal research was performed in accordance with an Animal Care and Use Protocol (ACUP ARS-2867) approved by the National Animal Disease Center's (NADC) Institutional Animal Care and Use Committee. At the end of study, all animals were humanely euthanized with the intravenous administration of a barbiturate (Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI) following the label dose (1 mL/4.45 kg).

Study 1: Sixteen finishing pigs born and raised at the NADC ranging from eight to nine months-of-age were randomly divided into 4 treatment groups (n=4/group): $10^{6.5}$, $10^{4.5}$, $10^{2.5}$, and $10^{0.5}$ $TCID_{50}$/mL inoculum. During the two-weeks animals were on study, they were individually housed in four ABSL-2 rooms. The same four rooms were used for each treatment group with cleaning, disinfection, and drying for 48-96 hours in between groups. Environmental swabs were collected weekly to monitor SVA in the environment. The $10^{0.5}$ treatment group was challenged first followed by successively higher titer inoculum groups ending with the $10^{6.5}$ treatment group. Pigs were challenged intranasally with 5 mL of inoculum split between both nostrils on 0 days post inoculation (dpi). Pigs were bled on 0, 3, 7, and 14 dpi and rectal swabs were collected daily from 0-14 dpi. Pigs were visually assessed for the formation of vesicular lesions on the coronary bands or snouts daily.

Study 2: Thirty piglets were farrowed at NADC from sows purchased from a commercial source. Sows were clinically free of lesions and were negative for neutralizing antibodies against SVA. At 24-72 hours after birth, piglets were weaned from the sow and blocking by litter were randomly allocated to 7 treatment groups. Six treatment groups (n=4/group) were inoculated

with $10^{5.5}$, $10^{4.5}$, $10^{3.5}$, $10^{2.5}$, $10^{1.5}$, or $10^{0.5}$ $TCID_{50}$/mL inoculum respectively. The seventh group (n=6) of pigs served as room contamination sentinels. Six ABSL-2 rooms, one for each challenge dose, were utilized for the study each containing five isolators and raised deck (Figure 1A). At weaning piglets were ear notched, bled, oral swabbed and rectal swabbed prior to placement in an individual isolator (-1 dpi) (Figure 1B). Pigs were given 24 hours to acclimate to feeding bowls and milk replacer. During feeding, piglets were fed one at a time by opening the feeding port, pouring in milk replacer closing the port before moving to the next isolator. The sentinal pig was always fed last. Piglets were challenged orally with 2 mL of inoculum on 0 dpi and thereafter isolators were only opened to provide milk replacer through the feeding port. On 6 dpi, piglets were bled, oral swabbed and rectal swabbed as they were individually removed from the isolators (6 dpi) and placed as a group (n=5) onto the raised deck in the room. Serum and rectal swabs were also collected on 10 and 14 dpi.

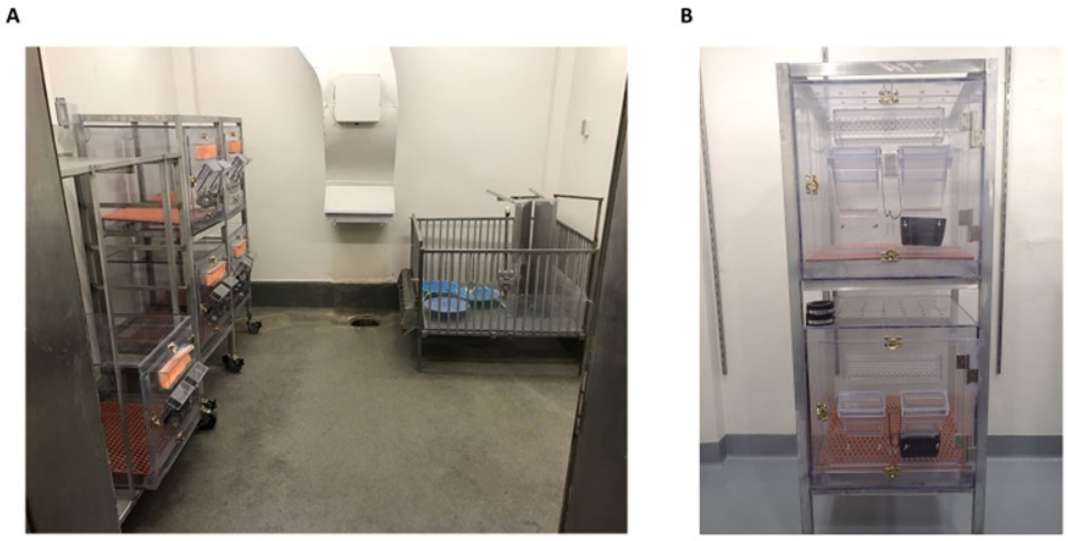

Figure 5.1: Housing for neonatal pig infectious dose study. A) Room set up for each challenge dose. Five individual housing units with a raised deck for subsequent group housing. B) Each individual unit has a small port used for feeding and holes in the unit for air circulation were covered with filter paper.

All animals were observed daily for clinical signs including lameness, lethargy, inappetence, and diarrhea. Serum was harvested from serum separator tubes (BD Vacutainer®, Franklin Lakes, NJ) used to collect blood. Oral, rectal, and environmental swabs were collected using a sterile polyester tipped applicator (Puritan Medical Products, Guilford, ME), immersed in 3 mL of MEM. Samples were stored in a -80°C freezer for future testing.

SVA nucleic acid extraction and quantification

Serum and swabs were tested by real time reverse transcriptase polymerase chain reaction (RT-qPCR) as previously described (22). Briefly, RNA was extracted from samples using the MagMAX™ Pathogen RNA/DNA kit (Applied Biosystems) following kit instructions for sample type. Next, 5 µL of extracted product was added to 20 µL of the Path-ID™ Multiplex One-Step RT-PCR reaction master mix (Applied Biosystems) for fecal swabs or 20 µL AgPath-ID™ One step RT-PCR master mix (Applied Biosystems) for sera and oral swabs. The forward primer sequence was 5'-TGCCTTGGATACTGCCTGATAG-3', the reverse primer sequence was 5'-GGTGCCAGAGGCTGTATCG-3' and the probe sequence was 5'-CGACGGCCTAGTCGGTCGGTT-3'. Ct values greater than 35 were considered negative.

A plasmid containing the target region for the primers and probe was used as a standard for quantification. A 490 nucleotide region (313-803) from the 5' untranslated region (5'UTR) and protein L of SVV-001 was cloned into the plasmid vector pCI-neo between the restriction enzymes XhoI and MluI (Promega, Madison, WI). The plasmid was transformed into One Shot® Electrocomp™ cells (Invitrogen, ThermoFisher Scientific) for plasmid propagation on LB agar plates with 50µg/mL Kanamycin. A QIAGEN® Plasmid Maxi Kit was used according to manufacturer's instructions for plasmid purification. RNA was quantified with a NanoDrop spectrophotometer (ThermoFisher Scientific) and the average nucleic acid quantity from three readings was used to calculate genomic copies (GC).

Virus neutralization (VN) assay

The virus neutralization assay has previously been described (22). In brief, serum samples were heat-inactivated and serially diluted 1:4 (up to 1:4096) in MEM with four replicates. An equal volume of SVA/CAN/2011 diluted to ~200 $TCID_{50}$ was added to the serum and incubated for 1 hour. The virus-serum mixture was transferred to confluent 96-well plates of ST cells. Plates were microscopically evaluated for cytopathic effect (CPE) daily for 4 days. Titers were recorded as the reciprocal of the highest dilution of serum at which the CPE of the SVA isolate was not visible in 50% of the inoculated wells. Titers $\leq$16 were considered negative.

Results

In Study 1, the SVA isolate SVA/CAN/2011 was serially hundred-fold diluted to create four challenge inoculums for finishing pigs with theoretical titers ranging between $10^{6.5}$ to $10^{0.5}$ $TCID_{50}$/mL for the 10^{0} to 10^{-6} dilutions respectively (Table 1). Table 1 shows the back-titration results from the inoculum as well as the Ct value and GC/mL from RT-qPCR. Dilutions from 10^{0} to 10^{-4} had higher titers than the theoretical titer. The 10^{-6} dilution was undetected by $TCID_{50}$ assay. Samples were tested in triplicate and the Ct value and GC/mL were calculated as an average of the three wells. Inoculum Ct values ranged from 11.0-32.6 which corresponded to $9.99x10^{9}$ to 4080 GC/mL.

Table 5.1: Titers, PCR Ct values, and GC/mL of virus dilutions used to inoculate finishing pigs.

Inoculum	Dilution of stock virus	$TCID_{50}$/mL (theoretical)	$TCID_{50}$/mL (back titrated)	Ct	Genomic copies/mL
$10^{6.5}$	10^{0}	3,160,000	5,010,000	11.0	9.99E+9
$10^{4.5}$	10^{-2}	31,600	63,100	18.7	5.35E+7
$10^{2.5}$	10^{-4}	316	1,260	25.6	4.75E+5
$10^{0.5}$	10^{-6}	3.16	0	32.6	4.08E+3

All four pigs in the $10^{6.5}$ treatment group replicated and shed SVA (Table 2). Rectal swabs were positive for SVA starting on 1 dpi with peak genomic copies observed on 6 dpi (Figure 2A). All animals had multiple positive swabs throughout the study with an average of 7 positive swabs per animal. In contrast, the $10^{4.5}$ treatment group did not have positive rectal swabs until 4 dpi with a similar peak at 6 dpi, but quickly declined. Animals in this group only had 2 positive swabs on average. Both the $10^{6.5}$ and $10^{4.5}$ groups had a smaller second peak of viral detection at 10 and 9 dpi respectively. One animal in the $10^{2.5}$ group had a positive rectal swab on 7 and 8 dpi. No animals in the $10^{0.5}$ challenge group had a positive rectal swab.

Table 5.2: Summary of finishing pig PCR positive status for each sample type throughout the study and VN titer for 14 dpi serum.

Inoculum	Serum	Oral swabs	Rectal swabs	VN titer
$10^{6.5}$	4/4	4/4	4/4	1024, 1024, 1024, 4096
$10^{4.5}$	2/4	2/4	4/4	1024, 1024, 1024, 4096
$10^{2.5}$	1/4	0/4	1/4	≤16, ≤16, 64, 1024
$10^{0.5}$	0/4	0/4	0/4	≤16, ≤16, ≤16, ≤16

One animal from the $10^{6.5}$ group did not have a detectable viremia, but the other three animals had peak SVA RNA levels at 4 dpi sample (Figure 2B). Two animals in the $10^{4.5}$ group and only one animal in the $10^{2.5}$ group had a detectable viremia (Table 2). Oral swabs taken on the same days as the blood samples had very similar PCR results to the serum except that all four animals in the $10^{6.5}$ group had positive oral swabs, while no animals in the $10^{2.5}$ group tested positive (Figure 2C). The same two pigs in the $10^{4.5}$ group had positive serum and oral swabs. Finally, no animals in the $10^{0.5}$ treatment group had positive PCR results for serum or oral swabs.

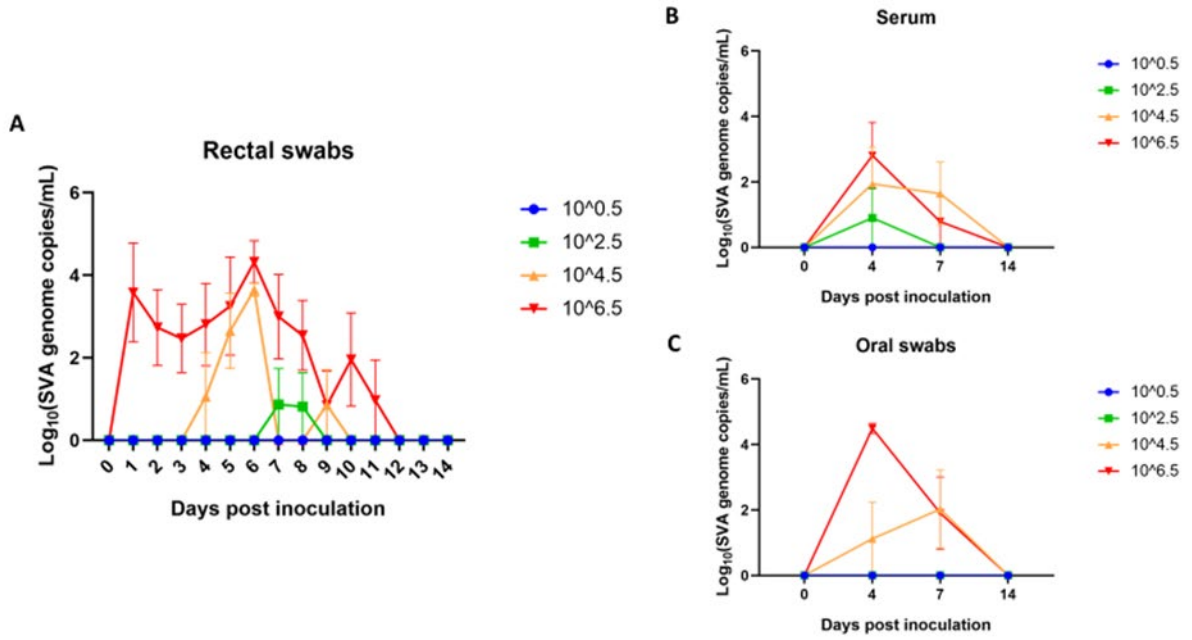

Figure 5.2:SVA infection dynamics measured by RT-qPCR in A) rectal swabs, B) serum, and C) oral swabs collected during the study. Each inoculum dose is color coded 106.5 (red), 104.5 (orange), 102.5 (green), and 100.5 (blue). Points on the graph represent the mean of the four animals and error bars and standard error of the mean.

Virus neutralization titers were determined for 0 dpi and necropsy serum (14-15 dpi). All animals had titers ≤16 on 0 dpi. All animals from groups $10^{6.5}$ and $10^{4.5}$ developed neutralizing

titers ranging from 1024-4096 (Table 2). Two pigs in the $10^{2.5}$ groups had titers of 64 and 1024 respectively, while the remaining two pigs had titers less than 16. No pigs in the highest dilution seroconverted.

In Study 2, SVA/CAN/2011 was serially ten-fold diluted to create six challenge inoculums for neonates with theoretical titers ranging between $10^{5.5}$ to $10^{0.5}$ TCID50/mL for the 10^{-1} to 10^{-6} dilutions respectively (Table 3). The 10^{-1} to 10^{-3} dilutions had higher titers than the theoretical titer, but 10^{-2}, 10^{-4}, and 10^{-5} were the same as predicted. Again the 10^{-6} dilution was undetected by $TCID_{50}$ assay. Inoculum Ct values ranged from 17.4-35.5 corresponding to $1.28x10^{8}$ to 570 GC/mL.

Table 5.3: Titers, PCR Ct values, and GC/mL of virus dilutions used to inoculate neonatal pigs.

Inoculum	Dilution of stock virus	$TCID_{50}$/mL (theoretical)	$TCID_{50}$/mL (back titrated)	Ct	Genomic copies/mL
$10^{5.5}$	10^{-1}	316,000	501,000	17.4	1.28E+8
$10^{4.5}$	10^{-2}	31,600	31,600	20.9	1.13E+7
$10^{3.5}$	10^{-3}	3,160	5,010	25.1	6.39E+5
$10^{2.5}$	10^{-4}	316	316	28.7	5.75E+4
$10^{1.5}$	10^{-5}	31.6	31.6	32.3	5.12E+3
$10^{0.5}$	10^{-6}	3.16	0	35.5*	5.70E+2

* One of the three wells was undetermined so the value is an average of two wells.

Due to the piglets being moved from individual animal housing to group housing on a deck, the 6 dpi samples collected as the animal was taken out of individual housing were the only

samples used for PCR determination of infection status. In addition, the VN assay was performed on 10 dpi serum assuming piglets infected from pen mates once combined would not have enough time to develop a robust neutralizing antibody response. All four pigs in the $10^{5.5}$ treatment group were viremic, had detectable SVA in oral and rectal swabs, and seroconverted (Table 4). Only 2 pigs in the $10^{4.5}$ treatment group had SVA positive serum and swab samples and a neutralizing antibody response. Both the $10^{3.5}$ and $10^{2.5}$ treatment groups only had 1 pig that had SVA positive samples; although, the pig in the $10^{3.5}$ group had a neutralizing antibody response while the pig from the $10^{2.5}$ group did not. All pigs in the $10^{1.5}$ and $10^{0.5}$ treatment groups tested negative for SVA and did not seroconvert. In addition, all the room control pigs were negative for SVA when removed from individual housing.

Table 5.4: Summary of neonate PCR positive status for each sample type on 6 dpi and VN titer for 10 dpi serum.

Inoculum	Serum	Oral swabs	Rectal swabs	VN titer
$10^{5.5}$	4/4	4/4	4/4	64, 256, 256, 1024
$10^{4.5}$	2/4	2/4	2/4	≤16, ≤16, 256, 1024
$10^{3.5}$	1/4	1/4	1/4	≤16, ≤16, ≤16, 1024
$10^{2.5}$	1/4	1/4	1/4	≤16, ≤16, ≤16, ≤16
$10^{1.5}$	0/4	0/4	0/4	≤16, ≤16, ≤16, ≤16
$10^{0.5}$	0/4	0/4	0/4	≤16, ≤16, ≤16, ≤16

Samples were also collected on 10 dpi and 14 dpi from all piglets while group housed to observe infection dynamics within each challenge group. All piglets including room controls from the $10^{5.5}$, $10^{4.5}$, $10^{3.5}$, and $10^{2.5}$ treatment groups were PCR positive in either serum or rectal swab collected on 10 dpi. By 14 dpi, viremia was detected in less animals, although most

animals in these groups still had positive rectal swabs. All piglets in the $10^{1.5}$ and $10^{0.5}$ treatment groups including room controls remained negative.

Tables 5 and 6 summarize the inoculum data with pig infection data. In finishing pigs inoculated with hundred-fold dilutions of SVA, the smallest dose that infected pigs was 1,260 $TCID_{50}$/mL (6,300 $TCID_{50}$/pig). In this group, SVA was only detected in samples from one pig, but a neutralizing antibody response was detected in two pigs. This inoculum had a PCR Ct value of 26.5 and the next dilution that did not infect pigs had a Ct value of 32.6. In neonates inoculated with ten-fold dilutions of SVA, the smallest dose to infect piglets was 316 $TCID_{50}$/mL (632 $TCID_{50}$/pig). Although only one pig had PCR positive samples and had not developed a neutralizing antibody response by 10 dpi, the remaining piglets in the group became infected when the piglets were co-mingled. This inoculum had a PCR Ct value of 29.7 while the following dilution that did not infect pigs had a value of 33.2.

Table 5.5: Summary of SVA infection outcomes in finishing pigs by PCR and VN assays.

$TCID_{50}$/mL	$TCID_{50}$/pig	Ct	PCR positive	VN positive
5,010,000	25,550,000	11.0	4/4 (100%)	4/4 (100%)
63,100	315,500	18.7	4/4 (100%)	4/4 (100%)
1,260	6,300	25.6	1/4 (25%)	2/4 (50%)
0	0	32.6	0/4 (0%)	0/4 (0%)

Table 5.6: Summary of SVA infection outcomes in neonatal pigs by PCR and VN assays.

$TCID_{50}$/mL	$TCID_{50}$/pig	Ct	PCR positive	VN positive
501,000	1,002,000	18.3	4/4 (100%)	4/4 (100%)
31,600	63,200	21.9	2/4 (50%)	2/4 (50%)
5,010	10,020	26.1	1/4 (25%)	1/4 (25%)
316	632	29.7	1/4 (25%)	0/4 (0%)
31.6	63.2	33.2	0/4 (0%)	0/4 (0%)
0	0	36.5	0/4 (0%)	0/4 (0%)

Discussion

The main goal of this study was to determine the minimum infectious dose of SVA in both finishing pigs and neonatal pigs. Due to the size of market-weight pigs and the nature of the study, only one pig could be housed in a room at a time; therefore, only 4 hundred-fold dilutions were tested. The size of neonates and the ability to individually house all piglets for one dilution in the same room allowed for a greater range of dilutions to be tested, 6 ten-fold dilutions. Therefore, even though the minimum infectious dose of market weight pigs (1,260 $TCID_{50}$/mL) was a higher titer than neonates (316 $TCID_{50}$/mL), it does not necessarily mean that neonatal pigs have a lower threshold. Now that this study has helped narrow the window of the minimum infectious dose in finishing pigs, further research with ten-fold dilutions could provide a more precise estimate.

None of the market-weight pigs that replicated virus and seroconverted developed vesicular lesions in this study. The same isolate given to 5-month-old pigs at a dose of ~$1x10^7$ $TCID_{50}$/mL did result in the development of lesions in 6/8 pigs (Buckley et al., unpublished). It

remains unclear why some animals develop lesions after exposure to SVA, while others do not. Although it was not observed in this study, one contributing factor could be inoculum dose. Most animals in experimental infection studies develop lesions, which could be attributed to the high doses of virus typically used for inoculation (10^7-10^8 $TCID_{50}$) versus what may be lower levels during natural exposure (9-12, 23, 24). Reports of the incidence of vesicular lesions in the field have ranged from 10-90% of animals on affected farms (6, 25-27). In addition, lesion development may vary based on pathogenicity of the viral isolate. A recent study compared the pathogenesis of two Chinese SVA isolates with a 96% nucleotide identity (29 AA changes). The 2016 isolate most similar to contemporary Canadian isolates did not result in any clinical disease, while the 2017 isolate similar to US strains did result in vesicular lesions in finishing pigs (28).

SVA was first detected in rectal swabs of finishing pigs from the $10^{6.5}$ group on 1 dpi with the $10^{4.5}$ group following on 4 dpi and finally the $10^{2.5}$ group on 7 dpi. In addition, the duration of PCR positive rectal swabs was correlated with dose as supported by the $10^{6.5}$ group shedding for 12 days, the $10^{4.5}$ group around 7 days, and the $10^{2.5}$ group for 2 days. Some of these trends were also observed in the PCR result from serum and oral fluids. These PCR results support the SVA dose an animal is exposed to may affect the infection dynamics including viral replication and shedding. The inoculum dose and route of exposure of FMDV in swine affects the incubation period and infection dynamics with greater doses shortening the incubation period and decreasing time to clinical signs (29-31). Therefore, biosecurity measures in the field to decrease viral exposure could aide in reducing the severity of infection and spread on the farm.

The four rooms housing finishing pigs in Study 1 were reused for each challenge dose starting with the highest dilution ($10^{0.5}$) and finishing with the lowest dilution challenge group

($10^{6.5}$) due to animal housing constraints. In between challenge groups, rooms were washed down, treated with Virkon™ S and allowed to dry twice, and rinsed with water. PCR negative environmental swabs of gating in the room supported the efficacy of the cleaning and disinfection process for rooms between usage. Other disinfection produces including accelerated hydrogen peroxide and bleach have shown efficacy against SVA (32, 33). A positive environmental swab from a room housing a pig in the $10^{6.5}$ group occurred right after the peak of SVA detection in rectal swabs; however, the remaining samples all tested negative suggesting there was not a high load of SVA in environment of these rooms. One explanation for lack of SVA detection in the environment of this study could be individual animal housing and is not representative of the load that may be present in a barn filled with infected animals.

Field reports from SVA affected breeding herds have described increased neonatal mortality in piglets during the first week of life and the syndrome was termed epidemic transient neonatal losses (ETNL) (5, 25, 27, 34). Clinical signs observed included lethargy, wasting, and diarrhea with cases in Brazil also reporting neurologic signs and vesicular lesions (15, 25). Most cases of neonatal mortality in the US did not observe significant gross or histologic lesions in affected piglets (34). In contrast, Brazil reported histologic lesions including interstitial pneumonia, myocarditis, glossitis, atrophy of intestinal villi, ballooning degeneration of the transitional epithelium in the bladder, and encephalitis (15). Immunohistochemistry identification of SVA in histologic lesions of the bladder, intestines, and central nervous system plus PCR testing to rule out other viral agents provided evidence that SVA was the causative agent for these lesions in neonates (13, 35). Piglets in Study 2 were between 2-4 days-of-age when inoculated with SVA and the only clinical sign observed some piglets across all challenge groups was soft stool during the first week after infection. Although SVA cannot be ruled out in

the pigs that were PCR positive for SVA, the change of diet from sow's milk to milk replacer likely played a role in the change in stool consistency. Thus, neonatal mortality due to SVA infection in breeding herds has yet to be experimentally reproduced and further research should be performed to better understand the contributing factors to ETNL.

Dose effect on acute infection dynamics in the neonates could not be evaluated in this study since small ports on the cages were only opened for feeding during individual housing. Cage doors were not opened to sample animals due to the risk of virus contamination in the room and exposure of animals to virus beyond the dose received during inoculation. The negative PCR status of the sentinel pig in each challenge room supported that neonates were only exposed to the inoculum dose of SVA. The first samples collected from the neonates was on 6 dpi when animals were removed from individual housing and those were used to determine PCR positive status. PCR data collected after 6 dpi could not be used for infection status since it could be confounded by exposure to shedding from other infected animals in the group housing, but that information was critical to shedding light on the infectivity of positive pigs. For example, PCR results from the $10^{2.5}$ group showed that four pigs (3 inoculated and 1 room control) negative for SVA on 6 dpi became positive on 10 dpi after contact with the one PCR positive pig from 6 dpi.

Epidemiologic investigations conducted on SVA affected breeding farms subjectively assessed that indirect transmission of SVA through contaminated farm employees, livestock trailers or carcass removal equipment were likely routes of virus introduction (25). Environmental sampling to determine level of SVA contamination is most often tested by PCR. Limited information is available to correlate SVA PCR Ct values to infectious virus. In this study, the minimum infectious dose for finishing pigs had a Ct value of 25.6 and neonates 29.7. Again, only hundred-fold dilutions were tested on finishing pigs, so the true minimum infectious

dose may have a higher Ct value. Inoculum Ct values of 32.6 and 33.2 for finishing pigs and neonates respectively were not able to infect pigs. Environmental samples with Ct values around 32 or greater may not present a large risk for infecting pigs and spreading SVA.

This study determined the minimum infectious dose of a 2011 SVA isolate after intranasal inoculation in finishing pigs and oral inoculation in neonates. Differences in the dilution series and inoculation route could have contributed to differences in the minimum infectious dose between finishing pigs and neonates (29). Recent studies have focused on more natural routes of exposure to determine minimum infectious dose such as oral exposure using natural feeding and drinking behaviors and pig-contact exposure (31, 36). In addition, other SVA isolates such as more contemporary strains may have different infectious doses. Information from this work can be used in future research to make more precise estimates of the infectious dose SVA with other strains and exposure routes. Understanding the minimum infectious dose of SVA can help producers and veterinarians in the swine industry focus their disease control and biosecurity measures on areas that carry the most risk of exposure of high levels of SVA.

Acknowledgements

The authors thank Deb Adolphson and Sarah Anderson for technical assistance and Jason Huegel, Justin Miller, Keiko Sampson, Nate Horman, and Dr. Jean Kaptur for assistance with animal studies.

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CHAPTER 6. EFFICACY OF A SENECAVIRUS A INACTIVATED VACCINE

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Modified to be submitted to Vaccines

Abstract

Senecavirus A (SVA), commonly known as Seneca Valley virus (SVV) is a causative agent for vesicular disease in swine. It has been found across the globe including the United States, Brazil, and China. Clinical disease caused by this virus is identical to foot-and-mouth disease virus (FMDV). Since FMDV has the potential to cause severe economic consequences in FMDV-free countries, those countries are on high alert for signs of vesicles in swine and an investigation is performed to rule out the presence of FMDV if observed. In countries where SVA cases have continued to occur, investigations and testing can cause a burden on personnel and resources. The objectives of this study were to test the efficacy of a whole-virus inactivated SVA vaccine against challenge in nursery-aged pigs and mature sows, and to assess the protection of passive maternal immunity generated by immunized dams. Pigs were given two doses of the vaccine intramuscularly three weeks apart and challenged intranasally two weeks after the second dose. Non-vaccinated pigs challenged with SVA developed clinical signs of disease, replicated virus, and developed a neutralizing antibody response. Vaccinated pigs had robust neutralizing titers after two doses; and after challenge, did not develop vesicular disease

and had limited rectal shedding. Piglets suckling immunized dams and challenged with SVA at 3-6 days-of-age had neutralizing titers prior to challenge and did not replicate or shed virus. An efficacious vaccine could improve swine welfare and reduce the economic consequences of continued foreign animal disease investigations.

Introduction

Senecavirus A (SVA) was first discovered as cell culture contaminant in 2002 (1). Retrospectively, testing of swine samples at the National Veterinary Services Laboratory had previously discovered a similar picorna-like virus in swine samples dating back to the late 1980s from various locations in the Unites States (US) and clinical histories (2). The speculation of the cell culture contamination being due to porcine trypsin (3) and presence of this virus in swine samples lead to the hypothesis that swine were the natural host (2). Early attempts to experimentally inoculate pigs with the virus did not result in specific clinical disease though some reported the virus did replicate in pigs (4, 5).

SVA is a non-enveloped, single-stranded, positive-sense RNA virus (~7.2 kb) that is the only member of the genus *Senecavirus* in the family *Picornviridae* (1). Other swine viruses in the family *Picornaviridae* include foot-and-mouth disease virus (FMDV), swine vesicular disease virus (SVDV), and encephalomyocarditis virus. Of the picornaviruses, SVA has been found to be most closely related to cardioviruses (6). SVA has one large open reading frame that encodes a single long polyprotein cut by viral proteases in the standard L-4-3-4 format of picornaviruses resulting in 12 mature proteins: leader protein, VP4, VP2, VP3, VP1, 2A, 2B, 2C, 3A, 3B, 3C, 3D (1). The structural proteins, VP1-4, form protomers that make up the icosahedral capsid of the virion. It has a long 5’ UTR that contains an internal ribosome entry site with a secondary structure that most resembles classical swine fever virus (5).

Late 2014 and into 2015, Brazil had a spike in idiopathic vesicular disease cases and increased neonatal mortality documented in the first week of life (7). Samples collected from these farms tested PCR positive for SVA (8). Shortly after, the United States saw similar cases of vesicular disease and neonatal mortality attributed to SVA starting the summer of 2015 (9-11). Contrary to attempts in the past, multiple groups were able to experimentally reproduce clinical disease in pigs of various ages demonstrating that SVA is a causative agent for vesicular disease in swine (12-14). Subsequently, other countries including Canada (15), China (16), Colombia (17), Thailand (18), and Vietnam (19) reported vesicular disease cases due to SVA. Including historical isolates, SVA strains from around the world share a nucleotide similarity of 93% or greater (20).

There are multiple viruses that infect swine and cause vesicular disease including: SVA, FMDV, SVDV, vesicular stomatitis, and vesicular exanthema of swine. The clinical disease caused by each of these viruses is grossly identical and further diagnostics must be performed to differentiate the cause of disease (8). Differentiating the cause of vesicular disease in swine is important, because foot-and mouth disease (FMD) caused by FMDV is on the World Organization for Animal Health (OIE) list of notifiable diseases (21). For countries with FMD negative status, identifying a case of FMD would have severe economic ramifications including production loss, trade restrictions, and cost of regaining FMD-free status (22). With more FMD-free countries identifying SVA, there is also an economic impact of an investigation to rule out FMDV every time an animal with vesicular disease is identified. A vaccine for SVA could reduce the occurrence of SVA-related vesicular disease thus improving swine welfare and reduce the economic burden of an endemic vesicular disease in FMD-free countries.

Research groups have previously shown the efficacy of a live-attenuated vaccine (23) and an inactivated vaccine (24) for protection against clinical disease of SVA in weaned pigs and finishing pigs respectively. To date, there have been no published studies evaluating vaccines in mature animals, though many reported cases of SVA vesicular disease occurred on sow farms with vesicular disease in sows and increased neonatal mortality (7, 11). The objectives of this study were to evaluate efficacy of an inactivated SVA vaccine in weaned pigs and mature sows, and the protection provided by maternal immunity from immunized dams.

Materials and Methods

Ethics statement

The animal work in this study was performed under approved animal care and use protocols (ARS-2867, ARS-2019-793) by the National Animal Disease Center's Institutional Animal Care and Use Committee. All animals were euthanized at the end of the study with an intravenous administration of a barbiturate (Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI) following the label dose (1 mL/4.54kg).

Virus inactivation and vaccine formulation

A SVA strain isolated in 2015 from a clinical case of vesicular disease in pigs from Iowa (SVA/2015/IA) was propagated on swine testicular cells cultured at 37 °C and 5% CO2 in minimum essential medium (MEM) supplemented with 2.5% HEPES (Gibco). Twenty-four hours after cell cultures were inoculated, supernatant was collected and clarified by 0.2 μM filtration. Pre-inactivation the virus titer ranged between 8.6-8.9 log $TCID_{50}$/mL. Binary ethylenimine (BEI) in addition to formalin were used to ensure inactivation of the virus. Material was neutralized with sodium thiosulfate and sodium bisulfite. Inactivation was confirmed by multiple passages in cell culture.

Inactivated virus was mixed with an oil-in-water adjuvant at 12.5%. Antigen and adjuvant were mixed for 30 min at room temperature and stored at 4°C prior to use. Vaccine was mixed gently directly prior to being drawn up into syringes for administration.

Challenge viruses

A SVA strain was isolated from a clinical case of vesicular disease in finishing pigs from South Dakota in 2015 (SVA/SD/2015). The isolate was propagated on a swine testicular (ST) cell line cultured at 37 °C and 5% CO2 in Minimum Essential Medium (MEM, MilliporeSigma, St. Louis, MO) supplemented with 10% FBS (AtlantaBio, Flowery Way, GA), gentamicin (VetOne/MWI, Boise, ID) and L-glutamine (Life Technologies, Carlsbad, CA). The virus was passaged 4 times in cell culture and was diluted to a titer of $1x10^7$ $TCID_{50}$/mL with MEM. Titrations were calculated using the Reed and Meunch method (25).

A second SVA strain was isolated from piglet intestine from a farm experiencing vesicular disease in sows and an increased neonatal mortality (SVA/KS/2018). Again, this isolate was propagated on ST cells. The isolate was passaged 3 times in cell culture and was diluted with MEM into two stocks for inoculation: $1x10^7$ $TCID_{50}$/mL and $1x10^5$ $TCID_{50}$/mL.

Animal studies

Study 1: Forty-six pigs were farrowed on-site at the National Animal Disease Center (NADC) in Ames, IA and housed under ABSL-2 conditions for the experiment. At weaning, pigs were randomly ear tagged and assigned to 4 treatment groups: control (n=8), vaccinated (vax) (n=8), challenge (n=15), and vax+challenge (n=15). At three weeks of age, the vax and vax+challenge treatment groups were immunized intramuscularly in the right side of the neck with 2 mL of inactivated SVA (SVA/IA/2015) plus adjuvant. A second dose was given intramuscularly on the left side of the neck (2 mL) three weeks later. At 0 days post challenge (dpc) animals in the challenge and vax+challenge groups were intranasally challenged with 5 mL

of SVA/SD/2015 ($1x10^7$ $TCID_{50}$/mL) divided between both nostrils (Table 1). Pigs were bled on -35, -14, 0, 3, 5, 7, 14 and 21 dpc. Group oral fluids and individual rectal swabs were collected daily from 0-10 dpc and on 14 and 21 dpc from challenged animals. Animals were euthanized on 21 dpc.

Study 2: Twenty sows were purchased from a commercial source in the Midwest of the United States and transported to the NADC. All animals were clinically free of lesions at the start of the study. Sows were bred via artificial insemination and 18/20 sows were confirmed pregnant by ultrasound. The two open sows were placed in the vax+challenge group while the remaining sows were randomly assigned to 4 groups: control (n=3), vax (n=3), challenge (n=5), and vax+challenge (n=9). At -35 dpc (~70 days of gestation) sows in the vax and vax+challenge treatment groups were immunized intramuscularly in the right side of the neck with 2 mL of inactivated SVA (SVA/IA/2015) plus adjuvant. A second dose was given intramuscularly on the left side of the neck (2 mL) three weeks later. At 0 dpc (~104 days of gestation) animals were separated into ABSL-2 rooms based on treatment and intranasally challenged with 5 mL of SVA/KS/2018 ($1x10^7$ $TCID_{50}$/mL) split between both nostrils (Table 1). Challenged sows were bled on -35, -14, 0, 3, 7, and 14 dpc and rectal swabbed and observed for vesicular lesions on 0-3, 5, 7, and 14 dpc. All sows were euthanized between 28 and 30 dpc.

Study 3: Piglets born to control sows (n=3 sows, 41 piglets) and vax sows (n=3 sows, 24 piglets) from Part 2 were challenged orally with 2 mL of SVA/KS/18 ($1x10^5$ $TCID_{50}$/mL) while still suckling their dams (3-6 days of age) (Table 1). Piglets were ear notched for identification. They were bled on 0, 5, and 14 days post inoculation (dpi) and rectal swabbed on 0, 1, 2, and 5. In addition, piglets were observed daily for clinical signs.

Table 6.1: Experimental design of animal studies.

Study	Group	N	Vaccine[a]	Timing	Challenge	Dose
Study 1	Control	8	None		Sham	
	Vax	8	SVA/IA/2015	-35 and -14 dpc	Sham	
	Challenge	15	None		SVA/SD/2015	$5x10^7$ $TCID_{50}$
	Vax+challenge	15	SVA/IA/2015	-35 and -14 dpc	SVA/SD/2015	$5x10^7$ $TCID_{50}$
Study 2	Control	3	None		Sham	
	Vax	3	SVA/IA/2015	-35 and -14 dpc	Sham	
	Challenge	5	None		SVA/KS/2018	$5x10^7$ $TCID_{50}$
	Vax+challenge	9	SVA/IA/2015	-35 and -14 dpc	SVA/KS/2018	$5x10^7$ $TCID_{50}$
Study 3	Control sow litters	3			SVA/KS/2018	$2x10^5$ $TCID_{50}$
	Vax sow litters	3			SVA/KS/2018	$2x10^5$ $TCID_{50}$

a Combined with an oil-in-water adjuvant at 12.5%.

Vesicular lesions were scored with a 5-point system: each foot that displayed a vesicular lesion was given 1 point and a vesicle on the snout was assigned 1 point. An animal was given a score of zero if no lesions were identified. Scores were assigned on 0, 3, 5, 7, and 9 dpc/dpi. Total clinical score was calculated by adding up the scores for all pigs in the group. Finally, pigs were observed daily for clinical signs of lameness, lethargy, and inappetence.

Blood was collected in serum separator tubes (BD Vacutainer®, Franklin Lakes, NJ) and centrifuged to harvest serum. Fecal swabs were collected using a sterile polyester tipped applicator (Puritan Medical Products, Guilford, ME), immersed in 3 mL of serum-free MEM. Oral fluids were collected with a white cotton rope hung for a half hour at pig level in each pen containing all pigs in the treatment group. Liquid was manually squeezed from the rope into a plastic bag and poured into vials for storage. All samples were stored at -80°C for future testing.

SVA nucleic acid quantification

Serum, swabs, and oral fluid samples were extracted and tested by real time RT-qPCR as previously described (26). Briefly, RNA was extracted from samples using the MagMAX™ Pathogen RNA/DNA kit (catalog no. 4462359; Applied Biosystems). Next, 5 µL of extracted product was added to 20 µL of the Path-ID™ Multiplex One-Step RT-PCR reaction master mix (Applied Biosystems) for fecal swabs and oral fluids or 20 µL AgPath-ID™ One step RT-PCR master mix (Applied Biosystems) for sera and vesicle swabs. The forward primer sequence was 5'-TGCCTTGGATACTGCCTGATAG-3', the reverse primer sequence was 5'-GGTGCCAGAGGCTGTATCG-3' and the probe sequence was 5'-CGACGGCCTAGTCGGTCGGTT-3'. RNA copies were calculated based on a standard curve generated from a plasmid containing the target region, and Ct values greater than 35 were considered negative.

Virus neutralization (VN) assay

The virus neutralization assay has previously been described (26). In brief, serum samples were heat-inactivated at 56 °C for 30 min, serially diluted 1:4 (up to 1:4096) in MEM and repeated in quadruplicate. An equal volume of diluted challenge virus, SVA/SD/15 or SVA/KS/18, (~200 $TCID_{50}$) was added to the serum and incubated for 1 hour. The virus-serum mixture was transferred to 96-well plates of ST cells. Plates were microscopically evaluated for cytopathic effect (CPE) daily for 4 days. Titers were recorded as the reciprocal of the highest dilution of serum at which the infectivity of the SVA isolate was completely neutralized in 50% of the inoculated wells. Virus neutralization titers of ≤16 were considered negative.

Statistical analysis

Statistical analysis was performed by multiple t-tests without the assumption of consistent standard deviation (SD). Multiple comparisons were corrected using the Holm-Sidak method with alpha = 0.5. Statistical analysis and graphs were performed and prepared using GraphPad Prism Version 8.1.2 (332) software.

Results

Study 1

Two groups of animals were sham challenged. The control pigs did not develop clinical signs of vesicular disease, SVA RNA was not detected in any sample tested by PCR, and pigs did not seroconvert with neutralizing antibody titers of ≤16 at 21 dpc. In the vax group, no animals were observed with vesicular lesions and SVA RNA was not detected in tested serum, rectal swabs, or oral fluids after sham challenge. After the first dose of vaccine, 3/8 animals had titers greater than 16. Following two doses of vaccine, all animals (8/8) in the vax group developed a VN titer of 256 (21 dpc) (Figure 1A).

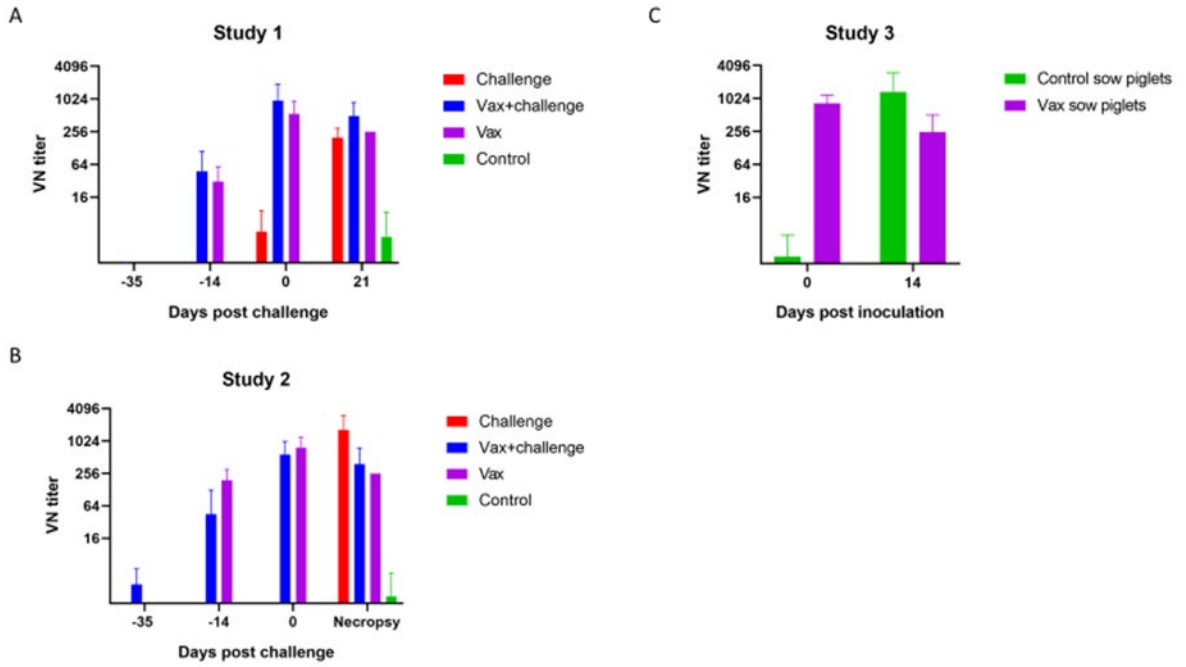

Figure 6.1: Virus neutralization titers. VN titers from serum collected in Study 1 against SVA/IA/2015 (A), and against SVA/KS/2018 in Study 2 (B) and Study 3 (C). The mean titer for the challenge group is represented by red bars, the vax+challenge group by blue bars, the vax group by purple bars, and the control group by green bars (A and B). Mean titers for piglets born to control sows is colored green and data from piglets born to vaccinated sows is colored purple (C). Error bars represent SD.

Two groups were intranasally challenged with SVA/SD/2015. In the challenge group, 12/15 animals developed vesicular lesions after challenge (Figure 2A). Lesions were first observed on 3 dpc with vesicle development on the coronary bands of 2 pigs and the peak number of animals observed with lesions occurred on 7 dpc. Snout lesions were not observed until 5 dpi with a total of 5/15 animals eventually developing erosions on the snout. The vax+challenge group did not develop any signs of vesicular disease following challenge.

SVA RNA was detected in rectal swabs by 1 dpc in both the challenge (12/15) and vax+challenge (13/15) pigs (Figure 2B). However, all animals in the vax+challenge group were negative by 2 dpc and remained negative for SVA by PCR in rectal swabs until the end of the

study. In contrast, SVA RNA was detected in rectal swabs from the challenge group from 1 dpc until the end of study (21 dpc) with 9/15 (60%) of pigs with a positive rectal swab at necropsy. There was a decrease in SVA detection in this group between 8 and 10 dpc, but genomic copies increased again by 14 dpc. SVA RNA was detected in serum in most animals (13/15) in the challenge group during the first week after challenge, with two animals that did not develop a measurable viremia (Figure 2C). No virus was detected in serum from the vax+challenge group. Similar to the serum PCR results, oral fluids demonstrated viral detection in the challenge group, but not in the vax+challenge group (Figure 2D). Viral levels in the oral fluids remained similar throughout the study.

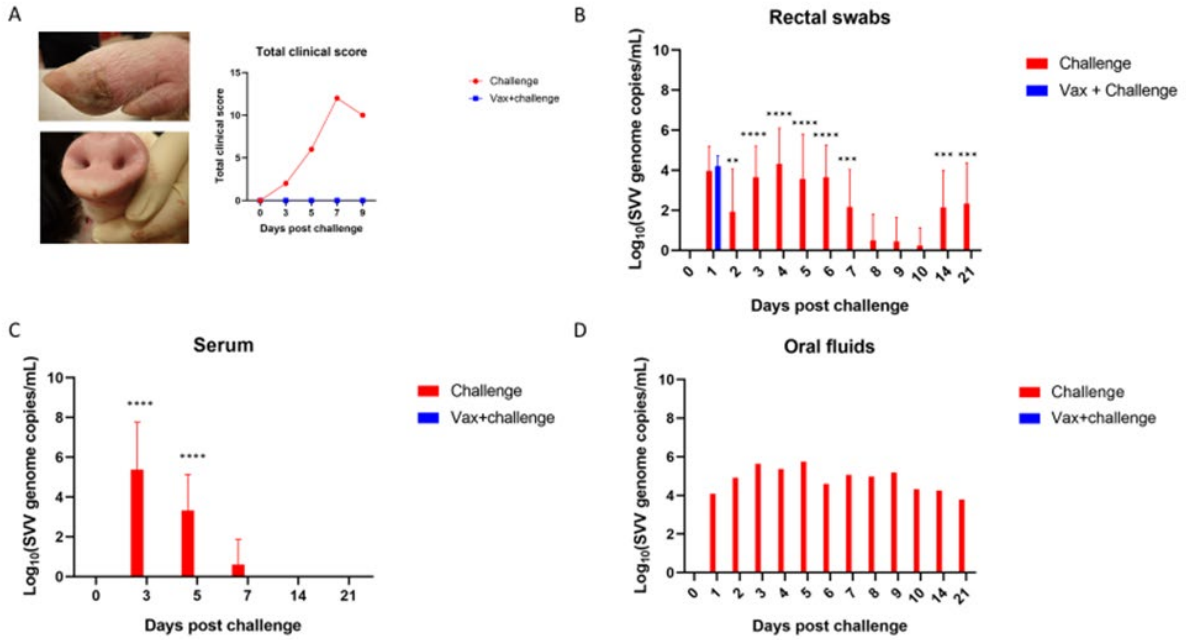

Figure 6.2: Clinical scores and virual load as determined by RT-qPCR in Study 1. A) Ruptured coronary band lesion observed in a challenged pig and a snout erosion observed on a challenge pig on 7 dpi. Animals were scored on a scale of 0-5. Scores were added up for each group and the total clinical score plotted. The mean log10(genomic copies/mL) for challenge pigs and vax+challenge pigs in rectal swabs (B) and serum (C). Error bars represent SD (**P < 0.01; ***P < 0.001; ****P < 0.0001). The log10(genomic copies/mL) of oral fluids collected from each group. Challenge pig data colored red and vax+challenge pig data colored blue.

All animals in the challenge group started with a VN titer ≤16 prior to challenge (0 dpc) and at 21 dpc VN titers ranged from 1:64 to 1:256 (Figure 1A), except one animal with a titer of 1:16. The pig with a titer of 1:16 was also one of the two pigs that did not have a detectable viremia; although, this animal did have positive rectal swabs throughout the study. Similar to the vax group, 7/15 animals in the vax+challenge group had VN titers greater than 16 after the first dose of vaccine. Prior to challenge, all animals in the vax+challenge group but one had titers greater than 16 following two doses of vaccine (0 dpc). At the end of the study (21 dpc), titers in the vax+challenge group ranged from 1:64 to 1:1024 which was similar to those animals in the challenge group (Figure 1A).

Study 2

Two groups of sows were sham challenged. Control sows did not develop clinical signs of vesicular disease, SVA RNA was not detected in any sample tested by PCR, and pigs did not develop a neutralizing antibody response with titers ≤ 16 at the end of the study. In the vax group, no animals were observed with vesicular lesions and SVA RNA was not detected in tested serum or rectal swabs after sham challenge. Animals in the vax group had VN titers ranging from 64 to 256 following one dose of vaccine (-14 dpc) and 256 to 1024 after two doses of vaccine (0 dpc) (Figure 1B).

Two groups were intranasally challenged with SVA/KS/18. In the challenge group, 2/5 animals developed vesicular lesions on their snouts (Figure 3A). No lesions were observed on coronary bands. The vax+challenge group did not develop any signs of vesicular disease following challenge. SVA RNA was first detected in two rectal swabs from both the challenge group (2/5) and the vax+challenge (2/9) group on 5 dpc (Figure 3B). The remaining rectal swabs were negative by PCR. SVA RNA was detected in the serum of two animals in the challenge group on 3 dpc (Figure 3C). In contrast, SVA was not detected in the serum from any animals in

the vax+challenge group. In general, low levels of SVA were detected by PCR after challenge and there was no significant difference between the two groups in rectal swabs or serum. In total, SVA RNA was detected by PCR in one sample, either serum, rectal swab, or vesicular swab in 4/5 animals in the challenge group and only 2/9 animals in the vax+challenge group.

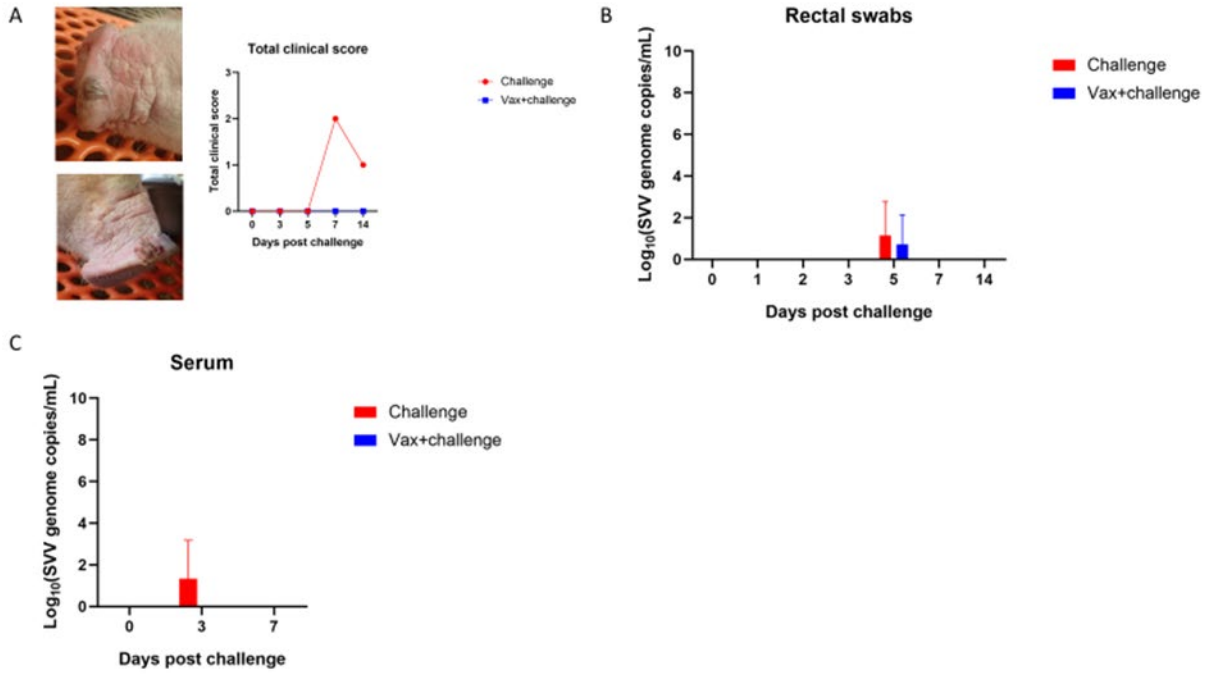

Figure 6.3: Clinical scores and viral load as determined by RT-qPCR in Study 2. A) Intact vesicle on the snout of a challenged sow at 8 dpi and the same lesion ruptured and healing on 14 dpi. B) Animals were scored on a scale of 0-5. Scores were added up for each group and the total clinical score plotted. The mean log10(genomic copies/mL) for challenge pigs and vax+challenge pigs in rectal swabs (C) and serum (D). Error bars represent SD. Challenged pig data colored red and vax+challenge pig data colored blue.

All animals started with a VN titer ≤ 16 prior to challenge (0 dpc). VN titers ranged from 1:1024 to 1:4096 in the challenge group at necropsy (Figure 1B). The vax+challenge group had VN titers ranging between 16 and 64 after one dose and from 64 to 1024 following two doses of vaccine (0 dpc). At the end of study, titers in the vax+challenge group remained between 64 and 1024. There was no significant difference in titers between the challenged and vaccinated animals at necropsy. Though animals in the challenge group had limited amounts of shedding

detected by PCR, all seroconverted after challenge and developed a robust neutralizing antibody titer.

Study 3

Control sows (n=3) and vax sows (n=3) farrowed and all piglets were challenged orally with SVA/KS/2018 around 3-6 days of age, while remaining with the sow. No clinical signs were observed in any of the piglets, except 2-3 piglets with a mild transient diarrhea in litters born to both control sows and vax sows. All piglets born to control sows were positive for SVA by PCR in serum samples collected on 5 dpi (42/42). A few piglets had positive 1 and 2 dpi rectal swabs; however, by 5 dpi 29/42 piglets had a PCR positive rectal swab. Piglets born from vaccinated sows did not have any PCR positive serum or rectal swabs after challenge (0/24).

The first four ear notched piglets were selected from each litter for VN testing on 0 and 14 dpi serum (Figure 1C). Prior to challenge, piglets tested from control sows had neutralizing titers ≤ 16. After challenge (14 dpi) piglets seroconverted with neutralizing titers from 256 to 4096. In contrast, piglets born to vaccinated sows had neutralizing titers ranging from 256 to 1024 prior to challenge due to suckling vaccinated sows. After challenge, VN titers decreased in most piglets and ranged from 16 to 1:1024, which follows waning maternal immunity.

Discussion

Vaccines play a critical role in the prevention and control of viral and bacterial diseases of swine (27). Inactivated whole-virus vaccines do not replicate in the host; therefore, they do not cause clinical disease and there is no potential to revert back to a virulent strain like live-attenuated vaccines. Inactivated vaccines have been proven to be safe and efficacious for viral diseases in swine (28). In this study, a whole-virus SVA inactivated vaccine was given in two doses to weaned pigs and sows and its efficacy against SVA challenge was evaluated. In

addition, maternal immunity provided by vaccinated sows was assessed by challenging suckling piglets.

Weaned pigs have previously been utilized for a SVA vaccine efficacy study and animals given two doses of an inactivated SVA vaccine and challenged with a heterologous SVA isolate developed viremia, shed virus detected by PCR in rectal, oral, and nasal swabs, and developed vesicular lesions (23). In contrast, vaccinated pigs in study 1 did not develop clinical disease, did not have a detectable viremia, and viral nucleic acid was only detected in rectal swabs on 1 dpc after challenge. It is likely this material was pass-through from the intranasal inoculation, but minor replication within the pig cannot be ruled out. Both studies used animals close in age and the inactivation process and adjuvant were similar, but the challenge virus in study 1 more closely matched the vaccine isolate with a 99.2% nucleotide identity of the whole genome compared to 97% nucleotide identity between the vaccine and challenge isolates in the previous study (23). In addition, Sharma et al. used a challenge dose of $1x10^{8.5}$ $TCID_{50}$ in 10 mLs given oronasally (23), while the challenge dose in this study was $5x10^{7}$ $TCID_{50}$ in 5 mLs given intranasally. Both the use of a more genetically dissimilar challenge isolate and a higher challenge dose could explain the difference in vaccine efficacies observed between these two studies.

Another study tested the efficacy of an inactivated SVA vaccine, but utilized finishing pigs rather than weaned pigs (24). In the other study pigs only received one dose of inactivated SVA vaccine prior to being challenged with $3x10^{9}$ $TCID_{50}$ and pigs were protected from the development of clinical disease and did not develop viremia after challenge, but further samples such as rectal/nasal swabs were not collected to look for viral shedding (24). The virus used for vaccination was the same virus used to challenge the pigs thus more likely to be effective than

versus a heterologous challenge. After two doses, vaccinated pigs in both study 1 and 2 had similar VN titers to non-vaccinated challenged pigs. Further work would need to be performed to evaluate if one dose would have been sufficient to provide protection.

Based on the success in Study 1, Study 2 was performed with mature sows since they may be the more likely candidate for vaccination. The cull sow network, which can include multiple collection points and trucking over large distances to group sows for slaughter plants, has been recognized as a disease transmission risk (29). In the US, sows with vesicular lesions have been observed on a regular basis when arriving at sow slaughter plants (J. Korslund, personal communication), thus vaccinated sows prior to entering the cull sow market could reduce or prevent the number of foreign animal disease (FAD) investigations occurring at sow slaughter plants. In 2018 there were 2,072 FAD investigations in the US with 1,592 involving vesicular disease conditions in pigs (30). These investigations are costly in terms of both time and resources to rule out FMDV every time a vesicle is observed on a pig.

This was the first time SVA/KS/18 was used to challenge swine after isolation and PCR results in mature animals showed limited viral replication. Based on results of the challenged sows, there was very little detectable virus in either the serum or swab samples collected, which makes it difficult to show reduction of viral replication and shedding between the challenge group and the vax+challenge group. Evidence that the challenge virus was recognized despite limited PCR detection was the robust neutralizing antibody response generated by animals in the challenge group. In addition, 2/5 challenge animals did develop vesicles on their snouts that contained high levels of virus by PCR. Experimental SVA challenge work with sows is limited, but another study using sows reported 4/5 sows developed vesicular lesions after challenge and field observations at sow farms experiencing SVA outbreaks have reported a large range in the

percentage of sows observed with vesicular lesions (11, 31, 32). Thus, it could be expected that not all sows develop lesions after challenge. Overall, the lack of lesion development in all nine animals of the vax+challenge group compared to lesion development in 2/5 challenged animals provides support that the vaccine protects against the development of vesicular disease in mature animals. Further evaluation of this vaccine in mature animals should be performed with another challenge strain to evaluate difference in viral replication and shedding.

Study 3 evaluated if the inactivated SVA vaccine given to sows could provide protection to nursing piglets challenged with SVA. In the field, there have been multiple reports of increased neonatal mortality in piglets less than 1 week of age that tested positive for SVA (11, 33, 34). Piglets have been reported with clinical signs including lethargy, diarrhea, and neurologic signs (35). Piglets challenged between 3-6 days of age after suckling control sows did not develop any clinical signs beyond a mild transient diarrhea in 2-3 piglets, which was also observed in piglets suckling vaccinated sows. Challenged piglets replicated virus and SVA was detected in both serum and rectal swabs. The pre-weaning mortality observed in this study was 12% for control sow litters and 4% for vax sow litters. The mean piglet pre-weaning mortality rate in commercial swine herds ranges between 10% and 20% (36) and the pre-weaning mortality during outbreaks in the field have been reported to be up to 60% (32). Although the control litters had a higher percentage of pre-weaning mortality compared to the vax litters, it still falls within the commercial norm. In addition, the larger litter sizes in the control sows litters (n=14, 14, 13) versus the vax sow litters (n=9, 9, 6) may have contributed to the higher pre-weaning mortality in this study.

Piglets born to vaccinated sows had VN titers measured in serum prior to challenge demonstrating that sows vaccinated around 70 days of gestation and boosted around 90 days of

gestation transferred immunity to their piglets. Negative PCR results supported that maternal immunity was able to protect piglets from SVA challenge. In contrast, piglets without immunity replicated virus and may be an important source of viral amplification during a SVA outbreak at a naïve farrowing facility.

In the US when a vesicle is observed on a pig, animal movement is stopped while trained personnel initiate a FAD investigation requiring sample collection for shipment to both a National Animal Health Laboratory Network lab and the Foreign Animal Disease Diagnostic Lab to rule out FMD. These FAD investigations and sample testing are costly to both the industry and the government, especially if SVA continues to remain endemic in the US swine herd. In addition, with the consistent occurrence of vesicular lesions in swine, veterinarians and producers in the swine industry may become compliant in assuming lesions are due to SVA infection. If FMDV were to enter the country and remain undetected for a period, the consequences could be devastating for not only the swine industry, but also animal agriculture and the economy. An effective SVA vaccine would be a valuable tool for the swine industry that could have a positive impact on welfare and reduce the economic burden of investigating vesicular disease cases.

Acknowledgments

The authors wish to thank Deb Adolphson and Sarah Anderson for their technical assistance and Jason Huegel, Justin Miller, Randy Leon, and Alyssa Dannon for their animal care and handling.

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CHAPTER 7. GENERAL CONCLUSION

Research Summary

Senecavirus A (SVA) was originally discovered as a cell culture contaminant in 2002, but similar picorna-like viruses had been found in swine samples in the US dating back to the 1980s. These isolates came from swine with various clinical histories, and experimental inoculation with swine did not result in specific clinical disease. SVA was detected sporadically until late 2014, when outbreaks of vesicular disease and neonatal mortality in Brazil had samples test positive for SVA. Similar cases appeared in the US in 2015, and experimental inoculation with contemporary SVA isolates were successful in reproducing vesicular lesions supporting SVA as a causative agent for vesicular disease in swine.

The main objective of this research was to better understand the pathogenesis of SVA in swine. The inability to reproduce clinical disease during initial attempts to experimentally infect swine with SVA led to speculation about the requirement of a co-factor or pathogenicity differences between strains. The early clinical history of lesions in pigs during marketing and traveling to fairs in the US drove our hypothesis that stress may exacerbate clinical disease in swine. To simulate stress, an immunosuppressive dexamethasone regime was administered to 9-week-old pigs prior to SVA inoculation. Clinical disease was similar between dexamethasone treated pigs and non-treated pigs. In addition, viremia, viral shedding, and neutralizing antibody response was similar between groups. Thus, this study supported that stress was not required for lesion development and provided information about infection dynamics that could be used by veterinarians and producers to develop strategies for diagnostics and control of SVA in the field.

To further explore infection dynamics and pathogenicity of different SVA strains, three historical isolates and three contemporary isolates were selected for comparison. There were

some apparent differences in timing of lesion development, viremia, and viral shedding, but all viruses replicated and caused vesicular disease in pigs. Serum from all pigs had cross-neutralizing antibodies against all viruses in the study; but, cross-neutralizing titers against historical isolates were lower than contemporary isolates. Sequence analysis of the six isolates in this study showed amino acid changes in prominent loop structures of the capsid that may play a role in the differences observed in cross-neutralizing titers. The study was the first to demonstrate that the original cell culture contaminant SVA isolate could cause vesicular disease in swine.

Experimental animal infections often use high titers of inoculum to ensure consistent replication of disease. Therefore, experimental infections may not be reflective of titers of virus swine are exposed to in the field. To better understand pathogenesis of SVA at different inoculum dosages and the infectious dose of SVA, serial hundred-fold dilutions and ten-fold dilutions of SVA were used to inoculate finishing pigs and neonates, respectively. In this study, with a 2011 SVA isolate, finishing pigs had a MID of $10^{3.1}$ $TCID_{50}$/mL and neonates $10^{2.5}$ $TCID_{50}$/mL. Although the MID for finishing pigs was higher in this study, the use of hundred-fold dilutions for inoculums provided a less precise estimate compared to the ten-fold dilutions used for neonates. This study also provided insight into the correlation of PCR Ct values with viral titers in cell culture and infectivity in swine. For example, swabs from vesicular lesions can contain titers of 10^6 $TCID_{50}$/mL, so contact with a vesicle could expose pigs to virus concentrations 3 logs higher then what is necessary to infect a finishing pig.

Vaccines play a critical role in the control and prevention of many veterinary diseases. The type of vaccine to be used is dependent on many factors that include safety, efficacy, and economy. Inactivated virus vaccines are often a safe and efficacious option that can be economic

to produce making this type of vaccine the initial choice to test. Two doses of a SVA inactivated vaccine prevented clinical disease and viremia and reduced viral shedding in rectal swabs in both weaned pigs and sows after challenge. In addition, passive maternal immunity from vaccinated sows appeared to provide sterile immunity to suckling pigs challenged with SVA. An effective vaccine could improve swine welfare and reduce the economic burden of FAD investigations.

These studies have provided critical information about the infection dynamics of various SVA isolates in swine and have demonstrated the efficacy of an inactivated vaccine against SVA challenge. If SVA remains endemic in FMDV-free countries, the burden of ruling out FMDV every time a vesicular lesion is observed in swine will remain. A better understanding of SVA pathogenesis, molecular evolution, and epidemiology is crucial to developing control and prevention measures for SVA in the global swine industry.

Future Work

Viral factors involved in pathogenicity of SVA and potential differences among strains are not understood. Future research utilizing reverse genetics could provide valuable information about individual protein functions and regions that may contribute to pathogenicity. Additional research to correlate PCR values, virus titration in cell culture, and infectivity with swine bioassay would help veterinarians make more educated decisions with PCR results. A follow-up infectious dose study in market-weight animals with ten-fold dilutions may provide a better estimate of the MID and help discern if there are age related differences in susceptibility to SVA infection. Finally, a vaccine study comparing the efficacy of one dose of an inactivated SVA vaccine to two doses would be beneficial to the swine industry. Not only is a one dose product easier to administer, but this study would also provide additional information about the neutralizing antibody titer required for protection.